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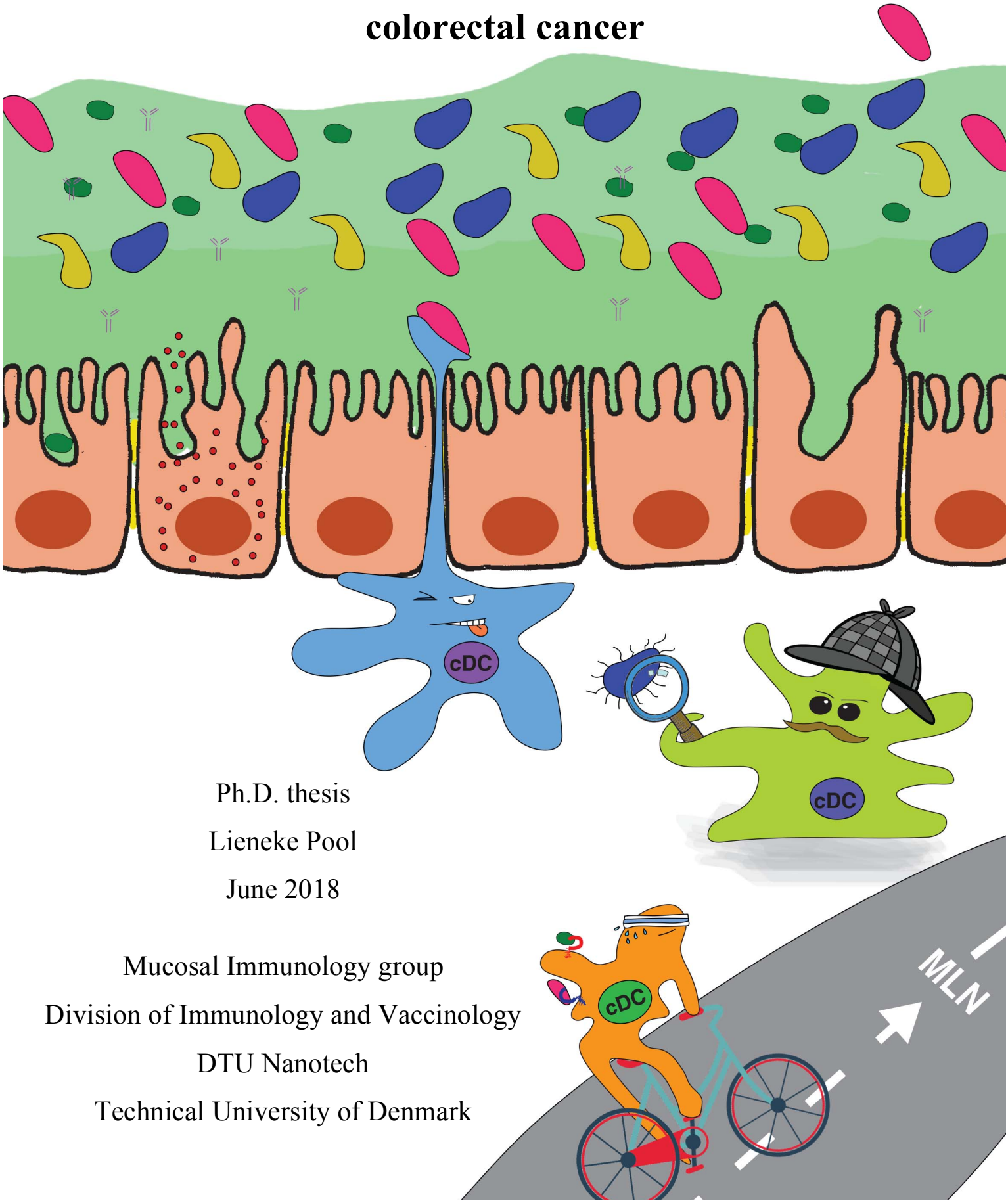

Ph.D. thesis

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To my family

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I **Lieneke Pool**, Aymeric Rivollier and, William W. Agace. IRF4 expression by cDC2 promotes the early generation of colitogenic T cells in T cell transfer colitis. 2018

In manuscript

II **Lieneke Pool** and, William W. Agace. IRF8 dependent cDC1 in AOM DSS induced colitis-associated cancer. 2018

In manuscript

Not included in this Thesis:

I Katarzyna M. Luda, Thorsten Joeris, Emma K. Persson, Aymeric Rivollier, Mimoza Demiri, Katarzyna M. Sitnik, **Lieneke Pool**, Jacob B. Holm, Felipe Melo-Gonzalez, Lisa Richter, Bart N. Lambrecht, Karsten Kristiansen, Mark A. Travis, Marcus Svensson-Frej, Knut Kotarsky and, William W. Agace. "IRF8 Transcription-Factor-Dependent Classical Dendritic Cells Are Essential for Intestinal T Cell Homeostasis" Immunity, volume 44, Issue 4, p860-874, 19 April 2016, http://dx.doi.org/10.1016/j.immuni.2016.02.008.

II Petra Holmkvist, **Lieneke Pool**, Karin Hägerbrand, William W. Agace and, Aymeric Rivollier "IL-18Rα-deficient $CD4^+$ T cells induce intestinal inflammation in the $CD45RB^{hi}$ transfer model of colitis despite impaired innate responsiveness." European Journal of Immunology, June 2016, Page 1371-1382 https://doi.org/10.1002/eji.201545957

III Aymeric Rivollier., **Lieneke Pool**, Ulrika Frising, Kerstin Wendland and, William W. Agace "Retinoic acid signaling is required for the efficient differentiation of $CD4^+$ T cells into pathogenic effector cells during the development of intestinal inflammation."

In Manuscript

Popular science summary

Our intestinal tract works hard for us continuously, it helps us take up important nutrients, vitamins and minerals from the food we ingest. Some of these processes take place with the help of an estimated amount of 10^{14} "good" microorganisms such as bacteria that ferment fiber. The immune system that is present in the intestinal wall has the complex task to distinguish between the beneficial food derived molecules and "good" microorganism and detrimental pathogens. Failure to maintain this balancing act, known as homeostasis, in combination with environmental factors and genetic predisposition can lead to chronic inflammatory bowel disease (IBD) such as Crohn's disease and Ulcerative colitis. Moreover, patients with IBD may develop inflammation induced colorectal cancer (CRC) when they have a genetic predisposition or when IBD treatment fails.

Classical dendritic cells (cDCs) are an important part of the intestinal immune system. cDCs are specialized 'sensors' that can recognize patterns and molecules (known as antigens) that are derived from pathogenic bacteria, good bacteria, the body's own cells and food with the help of a broad spectrum of different receptors that these cDCs express. Upon recognition of antigens the dendritic cells can activate specialized T cells that can either help clear detrimental pathogens/ tumor cells or prevent an immune response towards beneficial bacteria/ food derived molecules. In the intestine 2 main types of cDCs are present: cDC1 and cDC2. cDC1s are important in activating T helper 1 (Th1) cells that produce IFNγ which are important in the defense against parasitic and intracellular infections and in parts of the intestine they can induce regulatory T cells that possibly reduce responses to our own cells and food derived antigens. In other parts of the body cDC1s have been shown to activate Th1 cells and cytotoxic T cells with anti-tumor properties. cDC2 cells play a role in activating T helper 17 (Th17) cells and T helper 2 (Th2) cells, the former have different roles in maintaining homeostasis in the intestine while the latter are important for the defense against parasitic infections. To date however the role that cDC1s and cDC2s have in intestinal inflammation and CRC remains to be elucidated.

In the current thesis I assessed the role of cDC2s in an experimental model of T cell induced colitis and the role of cDC1s in an experimental model of inflammation

induced CRC. T cell induced colitis is thought to be dependent on a subset of pro-inflammatory Th17 cells. In manuscript I we showed that cDC2 cells play a role in the initiation phase of colitis development but were redundant once disease progressed. We suspect that the inflammatory environment attracts another type of inflammatory dendritic cells that is capable of inducing and maintaining pro-inflammatory T cells. Manuscript II describes that cDC1 cells have no significant role in inducing an anti-tumor response in a mouse model of inflammation induced CRC In the absence of cDC1 cells, despite a reduction in Th1 cells, mice developed tumors similar to mice that had normal amounts of cDC1 cells.

Dansk resumé

Vores fordøjelseskanal arbejder hele tiden hårdt for os, den hjælper os med at optage vigtige nærringsstoffer, vitaminer og mineraler fra den mad vi indtager. Nogle af disse processer finder sted ved hjælp af en estimeret mængde på 10^{14} "gode" mikroorganismer, som fx bakterier der fermenterer fibre. Immunsystemet som er til stede i tarmvæggen har den komplekse opgave at se forskel mellem de gavnlige molekyler fra fødevarer, samt "gode" mikroorganismer og skadelige pathogene organismer. Fejller denne balancegang, kendt som homeostasis, i sammenhæng med miljøfaktorer og genetisk prædisposition kan dette føre til inflammatorisk tarm sygdom (IBD) så som Crohn's sygdom og ulcerativ colitis. Hertil kommer at patienter med IBD kan udvikle inflammationsinduceret colorektalcancer (CRC) når de har en genetisk prædisposition eller når IBD behandlingen fejler.

Klassiske dendritceller (cDCs) er en vigtig del af tarmens immunforsvar. cDCs er specialiserede "sensorer", som kan genkende mønstre og molekyler, der kommer fra pathogene bakterier, gode bakterier, kroppens egne celler og fødevarer (kendt som antigener), ved hjælp af et bredt spekter af forskellige receptorer som cDCs udtrykker. Ved antigengenkendelse kan dendritceller aktivere specialiserede T celler, som enten kan hjælpe med at fjerne pathogener/tumor celler, eller forhindre et immunrespons mod nyttige bakterier/molekyler fra mad. I tarmen findes to hovedtyper af cDCs: cDC1 og cDC2. cDC1 er vigtige i aktiveringen af T hjælper 1 celler, der producerer IFNγ, som er vigtig i forsvaret mod parasitter og intracellulære infektioner, og i dele af tarmen kan de inducere regulatoriske T celler, der formentlig reducerer responsen imod vores egne celler samt antigener fra fødevare. I andre dele af kroppen er det blevet vist at cDC1 kan aktiverer T hjælper 1 celler og cytotoksiske T celler med anti-tumor egenskaber. cDC2 celler spiller en rolle i aktiveringen af T hjælper 17 celler og T hjælper 2 celler, hvor de første har forskellige roller i vedligeholdelsen af homeostasis i tarmen, mens de andre er vigtige i forsvaret imod parasitinfektioner. Til dato er rollen som cDC1 og cDC2 spiller i tarminflammation og CRC dog stadig ikke klarlagt.

I denne afhandling vurderer jeg cDC2 rolle i en eksperimentel model af T celle-induceret colitis og cDC1 rolle i en eksperimentel model for inflammations-induceret

CRC. T celle induceret colitis menes at være afhængig af en subpopulation af inflammatoriske T hjælper 17 celler. I manuskript I viste vi at cDC2 celler spiller en rolle i initieringsfasen af colitisudvikling, men var overflødige i den udviklede sygdom. Vi mistænker at det inflammatoriske miljø tiltrækker andre typer af inflammatoriske dendrit celler, som er i stand til at inducere og vedligeholde proinflammatoriske T celler. Manuscript II beskriver at cDC1 celler ikke spiller nogen signifikant rolle i induceringen af anti-tumor responsen i en musemodel af inflammationsinduceret CRC. I fraværet af cDC1 celler, selvom der er en reduktion i T hjælper 1 celler, udvikler mus tumorer sammenligneligt med mus der har normale mængder af cDC1 celler.

Abbreviations

IBD inflammatory bowel disease

CRC colorectal cancer

cDC classical dendritic cell

Th T helper

LP lamina propria

GALT gut associated lymphoid tissue

RegIIIγ regeneration islet-derived protein IIIγ

TLR toll like receptor

IL interleukin

NOD2 nucleotide-binding oligomerization domain

IEC intestinal epithelial cell

PRR pattern recognition receptor

MAMP microbe associated molecular pattern

DAMP damage associated molecular pattern

LPS lipopolysaccharide

NLR NOD-like receptor

ENS enteric nervous system

ILC innate lymphoid cell

NK natural killer

Lti lymphoid tissue-inducer

MLN mesenteric lymph node

PP peyer's patch

SILT solitary isolated lymphoid tissue

FAE Follicle associated epithelium

SED subepithelial dome

GAP goblet cell associated passage

MHC major histocompatibility complex

TCR T cell receptor

MadCAM1 vascular addressin cell adhesion molecule 1

RA retinoic acid

CPR15 G-coupled receptor 15

CTL cytotoxic T lymphocyte

STAT signal transducer of activated T cells

ROR retinoic acid receptor related orphan receptor

Treg T regulatory cell

FoxP3 forkhead box protein 3

CSN conserved non-coding DNA sequence

IEL intraepithelial lymphocyte

HSC hematopoietic stem cell

BM bone marrow

MDP macrophage dendritic cell progenitor

M-CSFR macrophage colony stimulating factor receptor

Flt3 fms-like tyrosine kinase 3

CDP common DC progenitor

SIRPα signal regulatory protein α

XCR1 chemokine XC receptor 1

GM-CSF granulocyte-macrophage colony-stimulating factor

Id2 DNA-binding protein inhibitor-2

IRF8 interferon regulatory factor 8

BATF3 basic leucine zipper ATF-like transcription factor 3

KLF4 kruppel like factor 4

Notch2 receptor neurogenic locus notch homolog protein 2

CD crohn's disease

UC ulcerative colitis

GWAS genome-wide association studies

AOM azoxymethane

DSS dextran sulfate sodium

TNBS trinitobenzene solphonic acid

Scid severe combined immune deficient

MDP muramyl dipeptide

DEC-205 endocytotic c-type lectin receptor 205

Smad7 SMAD family member 7

CAC colitis associated colorectal cancer

ROS reactive oxygen species

APC adenomatous polyposis coli

COX-2 cyclooxygenase-2

IDO indoleamine 2,3-dioxygenase

5-ASA 5-aminosalicylate

NSAID non-steroid anti-inflammatory drugs

Chapter 1 Intestinal biology and immunity

1.1 The intestinal tract

The intestine is anatomically adapted to digest food and absorb nutrients, minerals and water. Starting with the stomach and ending at the anus, the intestine forms a continuous tube that is lined with a single layer of epithelial cells. The intestinal tract can be roughly divided in the small intestine and the large intestine, which are separated by the ceacum. The small intestine and the large intestine have marked structural differences primarily dictated by the their physiological functions. The surface of the small intestine is covered by finger-like projections called villi, which are covered by a "brush border" consisting of a multitude of membrane projections known as microvilli, which serve to increase the surface area for optimal digestion and nutrient uptake. In contrast, the surface of the colon is relatively flat, but harbors a larger community of commensal microbiota compared to the small intestine. These microbes play an essential role for our health, through for example the fermentation of fiber, and the production of important essential vitamins and metabolites. As will later be described, they are also required for the function and development of the immune system.

The intestinal wall is build up of several distinct layers; in closest contact with the lumen is the mucosa, followed by the submucosa, the muscularis mucosa (a thin muscle layer) and the outer serosa (fibrous tissue separating the intestine from the peritoneal cavity). The mucosa comprises of the lamina propria (LP) and the muscularis mucosa, and is covered by the single layered epithelium that forms a barrier between the intestinal lumen and the underlying tissue. The LP is made up of loosely packed connective tissue that contains the large majority of innate and adaptive immune cells in the intestine, although immune cells can also be found in the intestinal epithelium and serosa. The main immune effector sites of the intestine are the LP and the epithelium, whereas induction of immune responses primarily takes place in draining lymph nodes and gut associated lymphoid tissue (GALT). In this thesis work the main focus will be on examining the immune response in the colon LP and intestinal draining lymph nodes.

1.2 The challenges for the intestinal immune system

The total area of the intestinal surface is estimated to be $32m^2$ making it the body's largest surface area that is in contact with the outside environment [1]. The immune system of the intestine is continuously exposed to dietary and bacterial derived products and therefore faces unique challenges compared to other organs. The major function of the intestine is to take up nutrients from food (e.g. proteins, vitamins, lipids and carbohydrates) and in this process it is important that the immune system doesn't overreact to dietary protein antigens. Digestion of plant polysaccharides and certain other dietary substances is to some extent dependent on commensal microbiota that are found throughout the intestinal tract at an estimated total count of 10^{14}; these bugs also need to be tolerated [2, 3]. The mucosal surface has both physical and biological barrier functions in place to help prevent breaching of this layer by pathogens or the microbiota. Further, there is a continuous cross-talk between the immune system and the microbiota as well as between immune- and non-hematopoietic cells in the mucosa that are key to maintaining tissue homeostasis. The intestinal immune system comprises of a diverse population of innate and adaptive immune cells with differential functions that cooperate tightly to elicit appropriate immune responses. Moreover, immune cells within the intestine, including macrophages and dendritic cells are environmentally imprinted with unique properties that serve to help maintain tissue homeostasis. In the intestine appropriate responses broadly comprise: recognition, containment and elimination of pathogens and tolerance towards beneficial microbiota and food antigens. Breakdown of the balance between these distinct tasks can lead to chronic infections, inflammatory bowel disorders or food intolerances. Moreover, the rapid and continual renewal of the intestinal epithelium (with an estimated half life of 4-5 days), likely in combination with continual immune activation makes that especially the large intestine is at high risk of developing malignancies, often as a result of chronic inflammatory disorders [4-6]. In the following chapters I will give a broad overview of the mucosal barrier, the intestinal immune system and its sub-compartments.

1.3 The epithelial barrier

In addition to enterocytes that are specialized in nutrient uptake, the intestinal epithelium contains specialized secretory epithelial cells, including goblet cells, paneth cells and tuft cells that have diverse antimicrobial properties. Goblet cells secrete mucin glycoproteins, which form a mucus layer that covers the epithelial surface. In the human small intestine goblet cells comprise about 10% of all epithelial cells, this proportion gradually increases to about 25% or less in the distal colonic epithelium [3]. The colon has two structurally distinct mucus layers, a loose outer mucus layer that contains large numbers of commensal bacteria that utilize the mucus as an energy source, and an epithelium-attached inner layer that is denser and contains few bacteria [7]. The small intestinal epithelium, in contrast, is covered with a single mucus layer that is looser, but contains antimicrobial peptides such as defensins and regeneration islet-derived protein IIIy (RegIIIy) [8]. These antimicrobial peptides are produced by paneth cells, which are long-lived columnar epithelial cells that in contrast to other epithelial cells move down to the base of the crypts after differentiating from stem cells [8, 9]. Paneth cells are unique to the small intestinal epithelium and release antimicrobial peptides in response to interleukin-22 (IL-22), stimulation of Toll-like receptors (TLRs), nucleotide-binding oligomerization domain 2 (NOD2) or cholinergic nerves [4, 8, 9]. Lastly, tuft cells constitute only about 0.4% of the intestinal epithelium and are less well characterized, mainly by the lack of suitable identifying markers [10]. These cells have a "tuft" of microvilli extending into the lumen, sense the environment through taste chemosensory receptors and transduce sensory signals, similar to taste receptors that respond to bitter-, sweet-, and umami-tasting substance, via Trpm5 [11]. Although their function is largely unknown, they have recently been implicated in the sensing of protozoa and helminth infections to which they respond with production of IL-25 which in turn activates type 2 immunity [12, 13].

Intestinal epithelial cell (IEC) function depends on a continuous cross-talk between commensal microbiota and the epithelium. This cross-talk is largely regulated by pattern recognition receptors (PRRs) that recognize microbe associated molecular patterns (MAMPs) and molecular patterns associated with cell damage or death known as damage associated molecular patterns (DAMPs). MAMPs include

lipopolysaccharide (LPS), bacterial lipoproteins and lipoteichoic acids, flagellin, unmethylated CpG DNA of bacteria and viruses, double stranded RNA and single-stranded viral RNA [14]. DAMPs are endogenous molecules that are released upon cell damage that can consist of extracellular matrix components, intracellular mitochondrial or nucleus components or components that are released during autophagy [15]. Each PRR has specificity for a given DAMP or MAMP, but different cell populations based on the location and functionality of the cell can express them in various combinations. PRRs such as Toll like receptors (TLRs) and NOD-like receptors (NLRs) are selectively expressed in distinct IEC compartments, a mechanism that is thought to partially contribute to the intestinal immune systems ability to discriminate between commensal and pathogenic bacteria. TLR5 for example is expressed at the basolateral surfaces of IECs, while TLR 3, 7, 8 and 9 are expressed in intracellular endosomal organelles and NLRs are present in the cytoplasm of IECs [16]. With this strategic placement of PRRs IECs only recognize pathogenic bacteria that actively invade the epithelial barrier [17]. As such IECs can act as early sentinels that upon activation can secrete mediators such as cytokines and chemokines that act to recruit and activate innate immune cells in the underlying LP tissue [18, 19]. Moreover, tight-junction integrity and trans-epithelial permeability are regulated by commensal microbial signals, including Toll like receptor 2 (TLR2) dependent redistribution of tight-junction proteins to apical cell-cell contacts [20]. Finally, like paneth cells, enterocytes are capable of producing antimicrobial peptides such as RegIIIγ throughout the colon and small intestine [19]. Although the mechanisms are not completely understood, the regional differences in antimicrobial peptide production throughout the intestine is thought to influence the composition and localization of microbial communities [19].

Thus, the epithelial barrier is a network of specialized epithelial cells that collectively help to maintain barrier integrity and the beneficial microbiota but also serve to limit access of microbes to the underlying LP.

1.4 Innate immune cells in the intestinal lamina propria

The intestinal LP contains a large population of innate and adaptive immune cells that help maintain intestinal barrier integrity and serve important functions in immune surveillance. Innate immune cells act as sentinels and respond rapidly, within minutes

to hours, to challenge when needed. The innate immune system consists of leukocytes that include populations of monocytes, macrophages, granulocytes, innate lymphoid cells and dendritic cells (DC).

Mononuclear phagocytes (macrophages and DCs) have been most implicated in the uptake and presentation of antigen in the intestine. While the main function of DCs is antigen presentation (which will be described in more detail in chapter 3), intestinal macrophages are at least in steady state a non-migratory population and thus their antigen presenting function maybe limited to the presentation of antigen to adaptive immune cells within the LP. Intestinal macrophages serve important phagocytic roles including the degradation of microorganisms and dead cells and production of mediators that regulate epithelial function and local immunity. Intestinal macrophages are well adapted to the microbe rich environment they occupy. They are highly bactericidal and can engulf bacteria through phagocytosis, but in contrast to macrophages in other tissues intestinal macrophages don't produce pro-inflammatory mediators in response to ingestion of bacteria [21]. Instead they produce IL-10, which serves to prevent inflammation and promotes survival and expansion of $FoxP3^+$ regulatory T cells in the mucosa [22-24]. Intestinal macrophages lack activating receptors (e.g. FcαR, FcγR, FcγRIII and some complement receptors), and although they express some PRRs (i.e. TLRs and NLRs) they are less responsive to stimulation of these receptors [21, 25]. They instead express inhibitory receptors like IL-10R and receptors for TGFβ. IL-10 has been implicated in the regulation of the PRR unresponsiveness while TGF-β derived from intestinal stroma has been suggested to drive the regulatory state of intestinal resident macrophages [26-29]. IL-10R on macrophages is essential for intestinal homeostasis as mice lacking IL-10R on macrophages develop spontaneous colitis [30, 31]. Moreover tissue resident macrophages produce IL1β, which has been implicated in the maintenance of stable Th17 activity in the steady state [32].

Eosinophils and mast cells are part of a larger group of granulocytes that are characterized by the presence of granules in their cytoplasm. These cells are mostly associated with allergic responses and responses to parasitic worms. However, recent studies suggest that some of these granulocytes have more diverse functions in maintaining intestinal homeostasis. For example eosinophils, which are found

throughout the intestinal LP in steady state have been implicated in tissue repair in steady state and during intestinal inflammation [33, 34]. More recently eosinophils have been implicated, through the production of APRIL and IL-1β, in T-cell independent IgA class switching [35, 36]. Mast cells are also found throughout the healthy gastrointestinal tract where they produce mediators (e.g. histamine and tryptase) that regulate epithelial barrier integrity [37]. Histamine affects the function of blood vessels, smooth muscle contraction and mucus production by epithelial cells [37]. Mast cells also interact with the local enteric nervous system (ENS) through production of histamine, proteases and lipid mediators by which they communicate to the ENS for the stimulation of peristalsis for rapid expulsion of for example toxins or helminthes [38]. It is however not known if mast cells control peristalsis under homeostatic conditions [37]. Mast cells also express TLRs and are thus likely to have a role in host defense against microbes [37].

Innate lymphoid cells (ILCs) are a recently described innate immune cell type that is present in mucosal tissues. ILCs develop from common lymphoid precursors, and can be subdivided into ILC1, ILC2 and ILC3 cells, with an effector cytokine-profile and transcription factor-dependence largely analogous to Th1, Th2 and Th17 cells, respectively. In contrast to adaptive lymphoid cells, ILCs do not express an antigen receptor. Instead, they are thought to rapidly respond to cytokines produced in the tissue environment. Group 1 ILCs are characterized by their Th1 like-phenotype and consist of natural killer (NK) cells and ILC1s. While NK cells display cytotoxic activities they can also produce large quantities of IFNγ in response to IL-12 similar to ILC1s [39-41]. Group 1 ILCs are implicated in the protection against bacteria and intracellular pathogens such as *Salmonella enterica* [42, 43]. Croup 2 ILCs are characterized by their expression of GATA3 and production of Th2 associated cytokines [39, 41]. Group 2 ILCs play an important role in resistance to nematodes such as *Nippostrongylus brasiliensis* and control of eosinophil homeostasis [44-46]. They produce Th2 cytokines (e.g. IL-4, IL-5, IL-13) in response to IL-25 and IL-33 [41]. Lastly, group 3 ILCs arise from lymphoid tissue-inducer (LTi) like precursors that require RORγt for their development [47]. Group 3 ILCs can be found as IL-22 producing or IL-17 producing subsets, the first expresses NKp46 and is found primarily in the small intestine and the latter is found in higher numbers in the colon [48]. RORγt^{+} ILC3s are important in early defense against intestinal pathogens such

as *Citrobacter rodentium* [49, 50] and in resistance to epithelial injury and intestinal inflammation [51]. In more recent studies a subset of group 2 and 3 ILCs have been shown to express MHCII, which possibly expands the functional properties of ILCs to that of antigen presenting cells [52-54]. $MHCII^+$ ILC2s and T cell cross-talk contributed to their mutual expansion and cytokine production, this interaction was crucial for a type 2 immune response towards *Nippostrongylus brasiliensis* [54]. Moreover, $ROR\gamma t^+MHCII^+$ ILC3s have been proposed to induce apoptosis of activated T cells specific for commensal microbiota [52, 53]. This may be an additional mechanism by which the immune system maintains homeostasis and controls immune responses towards commensal microbiota.

Chapter 2 Intestinal immune activation compartments and adaptive immunity

Adaptive immune responses are initiated in inductive sites of the intestinal immune system while B and T cells perform their effector functions in so called effector sites. These inductive sites are organized lymphoid structures with B and T cell areas encapsulated in organized stromal tissue. Inductive sites include the gut draining mesenteric lymph nodes (MLN) and gut associated lymphoid tissue (GALT) consisting of Peyer's patches (PP) in the small intestine and ceacal and colonic patches in the large intestine, whereas solitary isolated lymphoid tissues (SILT) can be found throughout the intestine. While the MLN are typical lymph node structures, connected to the intestinal mucosa via afferent lymphatic vessels, GALT are lymphoid structures localized directly underneath the epithelium in the intestine. MLN and PP develop prenatally and contain distinct B and T cell areas. In contrast, SILT consists primarily of B cell follicles and develops after birth in response to dietary and microbial factors [55, 56]. Effector sites include the intestinal epithelium and LP, where lymphocytes primed at inductive sites carry out their function.

2.1 Routes and mechanisms of antigen uptake by dendritic cells

The proposed mechanisms by which DCs acquire luminal antigens are many, and depend on the site of antigen acquisition and type of antigen. GALT structures are covered by a specialized epithelium, the follicle associated epithelium (FAE) that contains microfold cells (M cells). M cells are of epithelial origin and act as the port of entry for particulate luminal antigens such as bacteria. Luminal contents are endocytosed and transported by endocytic vesicles within M cells to the subepithelial dome (SED), where they are transferred to underlying DCs [57, 58].

In the LP some MNP are found relatively close to the epithelium and it has been suggested that these cells could extend processes into the epithelium to capture antigen directly from the lumen [59, 60]. Although it initially was thought that these cells were DCs, there is now a growing consensus that these cells are in fact macrophages expressing low levels of CD11c, MHCII and high levels of CX_3C-Chemokine receptor 1 (CX_3CR1) [61]. It has been further suggested that the

macrophages are capable of transferring these captured antigens to DCs, via gap junctions, as they themselves are not able to migrate to adaptive immune induction sites during homeostasis [62, 63]. Moreover, colonic DCs were recently found to receive antigens via goblet cell-associated antigen passages (GAPs) [64] an event that was already known to occur in the small intestine [65]. Alternatively, some subsets of DCs have been shown to capture antigen directly from the lumen by penetrating the epithelial monolayer while forming tight junctions with surrounding epithelial cells to prevent breaching of the epithelial barrier [18, 66]. While many mechanisms of antigen uptake have been proposed, whether these different uptake mechanisms lead to the initiation of distinct adaptive immune responses remains unclear.

2.2 Immune activation in the intestinal draining lymph nodes

Upon capturing of antigen by DCs a cascade of intracellular events results in the presentation of processed antigen on major histocompatibility complex (MHC) class I or class II. This process leads to functional maturation of DCs, which prepares the DC for their prominent role as professional antigen presenting cells to mediate activation of naïve T cells. During this maturation process, DCs upregulate co-signaling molecules on its surface, of which the CD80/CD86 complex is the best characterized [67]. Furthermore, activation of DC within the LP upregulates expression of the chemokine receptor CCR7 [68], which is required for their migration via afferent lymphatics to intestine-draining mesenteric lymph nodes (MLN), and also drives DC localization into the LN T cell zones [69]. DCs present their antigen via MHCI to $CD8^+$ T cells and on MHCII to $CD4^+$ T cells. In Chapter 3 I will go deeper into this mechanism, where I also summarize our current knowledge about the differential roles of DC subsets in T cell activation. MHC:peptide-complex interaction with the corresponding T cell receptors (TCR), co-stimulatory molecules (e.g. binding of CD80/86 to CD28 complexes on the T cell surface) and activating cytokines ensure the priming of naïve T cells into $CD8^+$ cytotoxic or $CD4^+$ T helper cells. This process leads to clonal expansion of antigen-specific T cells and the generation of effector and memory T cells. During this process, T cells that are primed in intestinal inductive sites also acquire expression of homing receptors, allowing them, once in the circulation, to efficiently enter intestinal effector sites. Homing to the small intestine is dependent on the chemokine receptor CCR9, which binds to CCL25 that is constitutively expressed by small intestinal epithelial cells, and integrin $\alpha4\beta7$ whose

ligand mucosal vascular addressin cell adhesion molecule 1 (MAdCAM1) is constitutively expressed by vascular endothelium within the intestine [70-72]. Imprinting of small intestinal-homing molecules occurs preferentially in intestine-associated inductive sites, and is thought to be dependent on retinoic acid (RA) production by small intestinal-derived cDCs and local stromal cells [73, 74]. Although less clearly defined, recent studies have linked G-coupled receptor 15 (GPR15) to T cell extravasation to the colon, which responds to its proposed ligand GPR15L that is highly expressed in the human and mouse large intestine [75-77].

2.3 Immune activation in GALT

IgA is the most abundant class of antibodies found in the intestinal lumen, and is part of the first line of defense protecting the intestinal epithelium from enteric pathogens and toxins. Luminal IgA also has a key role in the maintenance of diversity in the gut bacterial community [78]. The main site of B cell differentiation into IgA producing plasma cells is the GALT [79]. Secretory IgA production is almost completely dependent on the presence of the microbiota that gets sampled into the GALT via M cells as described in chapter 2.1 [4]. In the GALT, naïve B cells undergo class switching and become IgA^+ B cells, with the support from T follicular helper cells, and acquire homing potential to the intestinal LP. This event can also occur independently of T cell help, *Mora et al.* showed that GALT-DC derived RA, and IL-6 and IL-5 work synergistically to induce class switching and imprint gut tropism in naïve B cells [80]. Activated IgA^+ B cells migrate into the MLN, where they further proliferate and differentiate into IgA secreting plasmablasts that via the circulation extravasate into the intestinal LP [78, 81]. In the intestinal LP IgA is transported via endocytosis to the intestinal lumen upon binding to the epithelial polymeric Ig recptor (pIgR) [82].

2.4 Adaptive immune populations in the intestine

The intestinal effector tissues, the epithelium and LP, contain the body's largest pool of T cells. The LP and epithelium contain both $CD8^+$ and $CD4^+$ T cells, although $CD8^+$ T cells preferentially reside within the intestinal epithelium. The large majority of these cells has an effector memory phenotype and is thought to derive from naïve conventional T cells that have been primed in intestine-associated lymphoid organs, as described in the previous paragraphs. The $CD4^+$ T helper population in the healthy

intestinal LP consists primarily of Th1, Th17 and FoxP3$^+$ regulatory T cells, with few if any Th2 cells whereas the CD8$^+$ T cells are primarily cytotoxic T lymphocytes (CTL).

2.4.1 T helper cells

Th1 cells are characterized by their production of IFNγ and are involved in cellular immunity against intracellular microorganisms and anti-tumor immunity [83, 84]. IL-12 and IFNγ promote the polarization of CD4$^+$ T cells into a Th1 phenotype through signal transducer of activated T cells (STAT)1 and STAT4 signaling [63, 83, 85]. This cascade activates the T-box transcription factor T-bet, which drives the differentiation program of Th1 cells [86, 87]. Natural killer (NK) cells and Th1 cells are the main sources of IFNγ in Th1 differentiation and in both cell types T-bet promotes the IFNγ production [88, 89]. Furthermore, recent studies found that IL-27 is able to induce Th1 differentiation through STAT1 signaling in the absence of IL-12 [90].

Th17 cells are present in higher numbers in the intestine compared to non-intestinal sites and the discovery of this subset has changed the classical Th1/Th2 hypothesis [91-93]. Th17 cells are characterized by the expression of IL-17A, IL-17F and IL-22 and have a protective role in infections with fungi and extracellular bacteria [94]. IL-6 and TGFβ stimulate a cascade that leads to STAT6 signaling which drives the differentiation of Th17 cells that is regulated by transcription factor retinoic acid receptor related orphan receptor gamma (RORγt) [95-97]. Although IL-23 is not involved in Th17 differentiation it is important in the maintenance of its (pathogenic) effector functions [95]. More recently IL1β has been indicated as a potential inducer of Th17 differentiation [32, 98]. The microbiota is key to the generation of intestinal LP Th17 cells as these cells are largely missing from the intestine of germ free mice [99-101].

2.4.2 T regulatory cells

T regulatory cells are, as their name suggests, important in regulating immune responses by promoting tolerance to self- and foreign antigens and in dampening effector T cell responses during clearance of infection to limit autoimmunity and immunopathology. The intestinal Treg population contains thymic-derived natural

Tregs (nTregs) and peripherally induced Tregs (pTregs), also known as induced Tregs (iTregs), both of which are characterized by their expression of the transcription factor forkhead box protein 3 (FoxP3). Intestinal pTregs are believed to be generated from naïve $CD4^+$ T cells in intestinal inductive sites, through the action of TGFβ and retinoic acid (RA) [102, 103]. Several studies have suggested that nTregs can be distinguished from pTregs by their expression of the ikaros family member Helios and trans-membrane protein neuropilin-1 [104-109], although Helios expression may not unambiguously separate natural from peripheral Tregs [110, 111]. In the intestine an additional subset of induced Tregs, termed Tr1, can be identified that is characterized by its abundant production of IL-10 and lack of Foxp3 expression [112]. These $Foxp3^-$Tregs are like other pTregs dependent on TGFβ and are found in high numbers in the small intestine but are rare in the colon [112].

$Foxp3^+$ pTregs accumulation in the colon, but not small intestine, appears at least partially dependent on the microbiota as germ-free mice have reduced numbers of colonic $Foxp3^+$ pTregs [113, 114]. Colonization of germfree mice with certain bacterial strains, most notably clostridia species leads to a strong increase of colonic pTregs cells but not nTregs [113-115]. Mice with reduced pTregs through deletion of RORc in $FoxP3^+$ Tregs mounted a stronger Th2 response to intestinal helminth infections [107], deletion of pTregs however also resulted in Th2 induced pathology upon oxazolone-induced colitis [107]. Furthermore, mice deficient in conserved non-coding DNA sequence 1 (CSN1), a promoter that is dispensable for nTregs but crucial for iTreg development, developed spontaneous Th2 induced intestinal pathology and altered microbial communities [116]. Luminal metabolites such as vitamin A derived RA, vitamin D3 as well as short chain fatty acids produced through the fermentation of dietary fibers by the microbiota [107, 115] also seem to promote the generation, maintenance and function of intestinal Tregs. In contrast to the colon, the majority of pTregs in the small intestine is dependent on dietary antigen [117]. Although germ free mice were devoid of pTreg in the colon, pTregs were present in the small intestine of these mice, but upon being fed an elemental diet devoid of dietary antigens pTregs gradually decreased in the small intestine as well [117]. Absence of dietary antigen resulted in increased intestinal allergic pathology upon oral OVA stimulation after OT-II transfer [117]. The importance of Tregs in intestinal homeostasis has been demonstrated in a large number of studies. Colitis induced by

the transfer of naïve $CD4^+CD45RB^{hi}$ T cells in *Rag* deficient mice could be prevented or reversed by co-transfer of $CD4^+CD25^+CD45RB^{hi}$ Tregs [118]. Moreover $ROR\gamma t^+$ pTregs showed enhanced suppressive capacity in T cell transfer colitis compared to $RORyt^-$ nTregs [108]. Furthermore depletion of $Foxp3^+$ Tregs resulted in autoimmune inflammation and colitis that could be overcome by adoptive transfer of $Foxp3^+$ Tregs [119].

2.4.3 T cells in the intestinal epithelium

Intraepithelial lymphocytes (IELs) are, at least in the mouse, a highly heterogeneous population, of predominantly $CD8^+$ T cells. $CD8^+$ IELs comprise of conventional $CD8\alpha\beta^+TCR\alpha\beta^+$ T cells (major population in humans) and unconventional $CD8\alpha\alpha^+$ IEL that express either the γδ or αβ TCR [120]. All IELs express the integrin αE(CD103)β7, that binds to E-cadherin on the basolateral surface of intestinal epithelial cells [121], promoting their localization, maintenance and function.

Unconventional IELs develop in the thymus through positive selection on self-antigen and migrate directly to the intestinal epithelium. They populate the epithelium already at birth and this occurs independently of the microbiota or recognition of othere exogenous antigens [122, 123]. CD8αα is thought to function as a negative regulator of the MHC-TCR activation complex which may confer their mostly quiescent behavior [124]. $CD8\alpha\alpha^+TCR\gamma\delta^+$ IELs have been implicated in several processes; they can condition and repair the epithelial barrier, control IEC cell growth and turnover but also provide front-line defense against pathogenic enteric pathogens [120] while $CD8\alpha\alpha^+TCR\alpha\beta^+$ IELs have a potent antigen-experienced cytotoxic effector phenotype with high affinity to self-antigens and are believed to be important in front-line defense against invading pathogens and the maintenance of epithelial barrier integrity [121, 125]. Both subsets are thought to contribute to protective immunity by killing infected or injured cells by their cytotoxic phenotype [120, 126, 127].

In contrast, conventional IELs like LP effector T cells are thought to encounter their cognate antigen in the LNs and subsequently migrate to the epithelium. Conventional $CD8\alpha\beta^+$ $TCR\alpha\beta^+$ IELs mainly express a cytotoxic effector memory phenotype, while $CD4^+TCR\alpha\beta^+$ IELs can embody all Th subsets [125]. Conventional IEL numbers

increase with age and are dependent on the microbiota [123]. Protection against viruses that have evaded $CD8\alpha\beta^{+}TCR\alpha\beta^{+}$ IEL recognition can be provided by a subset of $CD4^{+}$ IELs that develop a cytotoxic phenotype and, upregulate CD8α, these cells can be found in the epithelium as $CD4^{+}CD8\alpha\alpha^{+}TCR\alpha\beta^{+}$ T cells [128-130]. Most effector cytotoxic T cells carry out antigen specific killing of infected cells or neoplastic cells by inducing apoptosis via Fas-FasL, pore formation via perforin secretion or lysis by release of granzyme B [131].

Chapter 3 Intestinal dendritic cells

By the 1960s it was known that lymphocytes are the main players in adaptive immunity, it was however unknown how antigens stimulated these lymphocytes. In 1973 Ralph Steinman and Zanvil Cohn first described a 'large stellate cell' in their splenocyte preparations [132]. The cell was termed "dendritic" and in later studies it was demonstrated that these cells possessed the unique capacity to stimulate naïve T lymphocytes [133]. These first studies by Ralph Steinman were subject of discussion and disregard, mostly because of the inability of other groups to reproduce their lymphocyte stimulating properties and their similarities to macrophages that were discovered already in 1884 [134, 135]. In the mid 80s the importance of classical DCs (cDCs) in immunological processes started to become more widely appreciated [135]. Ralph Steinman was awarded the Nobel Prize in physiology or medicine in 2011 for his discovery of cDC, unfortunately 3 days after his passing.

cDCs are a minor cell population of hematopoetic origin that populate most lymphoid and non-lymphoid tissues. In peripheral tissues they reside in an immature state and are characterized by dynamic extensions that allow these cells to 'sweep' the environment and act as motile sensors, scanning the local environment for foreign and self-antigens. The identification of surface markers that are expressed on monocyte derived cells and not on cDCs, including CD64, F4/80 and MerTK has made it easier to more definitively identify cDCs in tissues [136, 137]. Currently cDCs in intestinal tissues are identified by their expression of MHCII, the integrin subunit CD11c and lack of monocyte/macrophage expressed high-affinity IgG receptor FcγR1, CD64 [137-139]. Initially all $CD11c^{+}$ cells were classified as cDCs, this included a subset of CX_3CR1^{hi} macrophages that was discussed in chapter 2.1, later studies however revealed that these cells were derived from the monocyte lineage and are now broadly classified as macrophages by their expression of CD64 [137, 140, 141]. Both monocyte derived cells that are especially present in inflamed tissue and a subset of cDCs express intermediate levels of CX_3CR1, while resident macrophages express high levels of CX_3CR1, which was identified by using CX_3CR1-GFP mice [140, 142, 143]. Across tissues cDCs can be subdivided in two main branches: cDC1 that express the chemokine XC receptor 1 (XCR1) and cDC2 that express the signal regulatory protein α (SIRPα) [144]. Depending on the tissue cDC1 and cDC2 can be

further subdivided based on additional markers; this will be discussed in more detail in chapter 3.3.

3.1 Dendritic cell ontogeny

cDC originate from pluripotent hematopoietic stem cells (HSCs) in the bone marrow (BM). The earliest progenitor that is committed to the mononuclear lineage is the macrophage dendritic cell progenitor (MDP) that are characterized by their expression of macrophage colony stimulating factor receptor (M-CSFR), fms-like tyrosine kinase 3 (Flt3) and high levels of hematopoietic stem cell growth factor receptor C-kit (CD117) [145]. MDPs give rise to common DC progenitors (CDPs) that are C-kit$^{int/lo}$Flt3^{+}M-CSFR^{+} and give rise to exclusively pre-cDCs and pre-plasmacytoid DCs (pre-cDCs) [145-147]. CDPs that lost their expression of M-CSFR are thought to predominantly give rise to pre-pDCs, whereas CDPs that gave rise to cDCs maintained their expression of M-CSFR [148]. Pre-cDCs are capable of entering the blood stream and migrate into peripheral tissues, and they moreover express cDC markers CD11c and Zbtb46 [147, 149-151]. The differentiation of pre-cDCs into either cDC1 or cDC2 lineage was believed to take place in peripheral tissue with their downstream phenotype and functionality shaped by the local microenvironment [147, 152]. Recent findings however indicate that many pre-cDC are committed to the cDC1 and cDC2 lineage already within the BM [153]. Single CDP cultured *in vitro* were found to preferentially develop into either cDC1 or cDC2 subsets [154]. Subsequent studies using single cell RNA sequencing and single cell clonal assays showed that BM pre-cDCs preferential cDC1 or cDC2 lineage commitment could be predicted based on differential expression of SiglecH and Ly6C; SiglecH^{-}Ly6C^{+} pre-cDC giving rise to cDC2, while SiglecH^{-}Ly6C^{-} cells being committed to the cDC1 lineage [153].

The differentiation of cDCs from HSCs is largely dependent on the cytokines Flt3 ligand (Flt3L), and granulocyte-macrophage colony-stimulating factor (GM-CSF) [151, 155]. GM-CSF is not crucial for cDC differentiation in the steady state as GM-CSFR deficient mice only have a minor reduction in cDC numbers, it has however been indicated to act synergistically with Flt3L in DC development [156, 157]. Flt3L and GM-CSF control the initial production and lineage diversification of cDC. The

specific factors that regulate the expression of receptors for these key cytokines are however not fully elucidated. Moreover, Flt3L has also been indicated to maintain cDCs in peripheral tissues [158]. As discussed below cDC1 and cDC2 undergo further phenotypic changes depending on the tissue in which they reside.

3.2 Intestinal dendritic cells

Recent years have seen major advances in our understanding of the complexity and functionality of intestinal cDCs. In addition to XCR1 and SIRPα, intestinal cDCs can be subdivided based on expression of the integrin alpha chains CD103 and CD11b [136, 159, 160]. $XCR1^+$ cDC1 are primarily $CD103^+CD11b^-$, while $SIRP\alpha^+$ cDC2 can be divided into $CD103^+CD11b^+$, $CD103^-CD11b^+$ cDCs [136]. In the steady state intestine a minor population of poorly defined $CD103^-CD11b^-$ cDC can also be found, which appears heterogeneous in that they contain both $XCR1^+$ and $SIRP\alpha^+$ cDCs [136]. cDCs are found throughout the intestinal LP, and within intestinal inductive sites. Within the LP the cDC1: cDC2 ratio is similar along the length of the intestine although $CD103^+CD11b^+$ cDC2s are found in higher frequencies in the small intestine while $CD103^-CD11b^+$ cDC2s are more common in the colon [4, 136]. Recent findings showed that $CD103^-CD11b^+$ cDCs can be further subdivided in $CCR2^-$ and $CCR2^+$ populations [161] suggesting further heterogeneity within the intestinal cDC2 compartment.

Similar to all lymph nodes, intestinal draining MLN contain cDC1 and cDC2 of distinct origins, a lymph node resident population that enter the lymph node directly from the blood, and a LP derived migratory population. It has been proposed that under steady state conditions these populations can be distinguished based on their expression of MHCII with lymph node resident cDCs expressing lower levels of MHCII [162, 163].

3.3 Intestinal dendritic cell ontogeny

The first realization of heterogeneity in cDC population came with the discovery of $CD8\alpha^+$ and $CD8\alpha^-$ cDCs in the spleen and thymus [164]. Almost 2 decades later our understanding has grown substantially but across tissues these 2 cDC subset are still recognized as the main branches of cDCs. We can now however further separate these

branches into subsets with distinct and overlapping functionality based on a broader panel of transcription factors and surface markers.

3.3.1 (Intestinal) cDC1 development

The development of intestinal cDC1 has not been as widely studied as intestinal cDC2 development. In this chapter I will therefore discuss factors that are identified to be involved in intestinal cDC1 development and factors that were shown to be involved in cDC1 development on other peripheral tissues but could possibly apply for intestinal cDC1.

Intestinal cDC1 development: The development of intestinal $CD103^+CD11b^-$ cDC1, as in other peripheral tissues, is dependent on the transcription factors interferon regulatory factor 8 (IRF8), DNA-binding protein inhibitor-2 (Id2) and basic leucine zipper ATF-like transcription factor 3 (BATF3) [159, 165, 166]. Here IRF8 is the key transcription factor that acts upstream of Id2 and BATF3, and is critical for the lineage commitment of cDC1. Mice with a specific deletion of IRF8 in $CD11c^+$ or $ZBTB46^+$ cells lack all intestinal $CD103^+CD11b^-$ cells [166, 167]. LN resident $CD8\alpha^+$ and lung tissue $CD103^+$ cDCs require 2 alleles of IRF8 whereas the development of $CD103^+CD11b^-$ cDCs appears unaffected in mice carrying only one copy of the IRF8 allele, indicating that dependence of cDC1 on IRF8 differs between tissues [166, 168, 169].

Peripheral tissue cDC1 development: IRF8 induces the expression of pan-cDC1 markers such as XCR1, and CLEC9A, while Id2 and BATF3 further enhance their expression [170, 171]. In a DC progenitor like cell line IRF8 expression has been shown to activate the expression of Id2 and BATF3 and facilitates the functional maturation of cDC1s by activating TLR signaling pathways [171]. Moreover Id2 and BATF3 expression alone were not sufficient for directing cDC1 development [171]. BATF3 acts later in the development and does not affect the development of CDPs or pre-cDCs, it has however been shown to be important for the cross-presenting capacity of cDC1s [165, 172]. Interestingly, during intracellular infections in $Batf3^{-/-}$ mice, cDC1s can develop independently of BATF3 [173]. It was shown that IL-12 production in response to IFNγ restored cDC1 numbers and $CD8^+$ T cell responses due to compensation by BATF [173]. IRF8, Id2, BATF3 and possibly other

transcription factors seem to have important roles in different stages of cDC1 development and act in a synergistic fashion to facilitate cDC1 development and functionality. Id2 is also expressed by all cDC subsets with the highest expression in cDC1s, it is thought that Id2 acts as a inhibitor of pDC-lineage specifying transcription factor E2-2 and thereby promoting the development of cDCs [170, 174].

3.3.2 Intestinal cDC2 development

Intestinal cDC2 appear far more heterogeneous than cDC1, and their characterization has been to some extent hampered by the fact that they share certain markers with monocyte-derived populations. Intestinal cDC2 cells express CD11b and SIRPα but lack expression of XCR1, DNGR-1 and CD8α [136, 175, 176]. Intestinal $CD103^+CD11b^+$ cDCs depend, at least in part on the transcription factors IRF4, kruppel like factor 4 (KLF4) and receptor neurogenic locus notch homolog protein 2 (NOTCH2) for their development and/or survival [177-179]. IRF4 deletion in $CD11c^+$ cells leads to a 50% reduction of $CD11b^+CD103^+$ cDCs in both small and large intestine and an almost complete reduction in the MLN [177]. The remaining $CD103^+CD11b^+$ cDCs in the small intestine in CD11c-Cre.*Irf4*$^{fl/fl}$ mice showed decreased survival in vitro, indicating that IRF4 is supporting survival of $CD103^+CD11b^+$ cDCs [177]. Moreover, CD11c-Cre.Irf4$^{fl/fl}$ mice displayed a reduction in $CCR2^+$ $CD103^-CD11b^+$ cDCs [161]. Recently the transcription factor zinc finger E box-binding homebox 2 (Zeb2) was found to be expressed on pre-cDC2s and cDC2s but was down regulated on pre-cDC1s and cDC1s [153, 180]. Zeb2 regulates commitment towards cDC2 by repressing Id2 [181]. In mice that lacked expression of Zeb2 on $CD11c^+$ cells $CD103^-CD11b^+$ cells were decreased in the small intestine but not the MLN whereas $CD103^+CD11b^+$ cells were unaffected [181]. Interestingly there seems to be a potential relationship between the cDC2 subsets, in mice lacking TGFβRII in CD11c cells $CD103^+CD11b^+$ cDC were dramatically reduced with a reciprocal accumulation of $CD103^-CD11b^+$ cDCs [182]. It was proposed that $CD103^-CD11b^+$ are immediate pre-cursors to $CD103^+CD11b^+$ cDCs and that this developmental stage is under control of TGFβ [182]. It however remains to be determined where in the developmental stage TGFβ signaling is required for the development of $CD103^+CD11b^+$ cDC2s. Different transcription factors and soluble factors might act in different stages of cDC2 development and due

to the heterogeneous nature of cDC2s the different transcription factors might work synergistically in the development of functionally different subsets.

3.4 Intestinal dendritic cell functionality

3.4.1 cDC1 functionality

cDC1s can present endogenous antigen, such as viral proteins that are degraded in the cytosol and transported to the endoplasmic reticulum from where they get loaded onto MHCI. Or they can cross-present, a process in which exogenous antigen gets phagocytozed and either gets degraded in phagosomes from which they are directly loaded on MHCI or the phagocytosed antigen is exported to the cytosol and is processed as described for endogenous antigens. This process of cross-presentation is important in protection to intracellular infections and in the elimination of tumor cells.

Studies using CD11c.cre.Irf8$^{fl/fl}$ and XCR1-DTA mice have identified an important role for cDC1s in intestinal adaptive immune homeostasis [166, 183]. These mice have reduced numbers of small intestinal LP CD4$^+$ and CD8$^+$ T cells and both studies found a major reduction in some subsets of small intestinal CD8$^+$ IELs [166]. *Ohta et al.* suggested that cross talk of XCR1 on cDCs and its ligand XCL1 on T cells was important for overall survival and maintenance of LP and IEL T cells [183]. XCL1 and XCR1 cross-talk enhanced survival of intestinal T cells by upregulating CD103 and CD62L and in turn continued XCL1 expression on T cells enhanced cDC maturation and CCR7 upregulation enabling their migration to MLNs [183]. Moreover migratory cDC1 in the MLN are the major source of retinoic acid (RA), which is a key inducer of CCR9 and α4β7 that enables activated T cell to home to the small intestine [73, 166]. Possibly, the reduced migration capacities of primed T cells resulted in a reduction of conventional CD8αβ$^+$ and CD4αβ$^+$ T cells in the LP [166]. However, the effect that absence of cDC1 cells has on the T cell homeostasis in the large intestine remains to be explored.

CD11c-Cre.*Irf8*$^{fl/fl}$ mice have normal proportions of Th17 and FoxP3$^+$ Tregs in the small intestine, while they completely lack Th1 cells [166]. In the colon Th17 cells were also unaffected in CD11c-Cre.Irf8$^{fl/fl}$ mice, while FoxP3$^+$ Tregs were slightly reduced and Th1 cells were absent [166]. This is consistent with other studies

showing that mice lacking cDC1s have an impaired response to colonic helminth infections, to infections with intracellular pathogens and have increased resistance to DSS induced colitis due to impaired Th1 responses or reduced IFNγ production [184-186].

Finally, *Esterhazy et al*. demonstrated that priming of FoxP3$^+$ pTregs in response to dietary antigens is reduced in *Zbtb46*-Cre.*Irf8*$^{fl/fl}$ mice, indicating that cDC1s are involved in the generation of pTregs [167]. Although the reduced pTreg generation in *Zbtb46*-Cre.*Irf8*$^{fl/fl}$ mice did not result in impaired tolerance towards dietary antigen, which may be dependent on nTregs that are possibly not affected in these mice.

3.4.2 cDC2 functionality

As pointed out in chapter 2.3 cDC2s are a far more heterogeneous population of cDCs compared to cDC1s, and the lack of a mouse model that can selectively deplete all cDC2s makes it more challenging to identify and compare functional properties of cDC2 subsets. However some of the existing mouse models may cause impaired functionality in all cDC2s without depleting all of them.

Studies using mice that lack expression of IRF4 or Notch2 on CD11c$^+$ cells or SIRPα$^{-/-}$ mice all show selective reduction in intestinal Th17 cells [161, 176, 177, 179, 187] and *huLangerin*-DTA mice, which lack CD103$^+$CD11b$^+$ cDC2s, have reduced numbers of small intestinal Th17 cells [188]. IRF4 has been indicated as an important promoter of MHCII antigen processing machinery in cDC2 [189]. Both the absence of CD103$^+$CD11b$^+$ and/or impaired MHCII antigen processing may lead to reduced amounts of Th17 cells in mice lacking IRF4 dependent cDC2s [175, 176, 189]. While in most of these models the underlying mechanisms of reduced Th17 number remains to be elucidated, one contributing factor might be that cDC2s produce IL-6 that may enhance the Th17 differentiation in the MLN [177]. Interestingly, mice that have specific deletion of *Klf4* in CD11c$^+$ cells have normal intestinal Th17 responses [178]. Indicating that *Klf4* only affects a subset of IRF4 dependent cDC2 cells that does not entail Th17 promoting cDC2s; in contrast CD11c-cre.Klf4$^{fl/fl}$ mice have impaired Th2 responses towards *Schistosoma mansoni* [178]. Consistent with this CD11cCre.Irf4$^{fl/fl}$ mice fail to induce a Th2 response towards parasitic worms *Nippostrongylus brasiliensis* or *Trichuris muris* [186, 190, 191]. While CD103$^+$CD11b$^+$ cDC2s were important in protective immunity towards *Schistosoma mansoni* in the small intestine,

CD103^{-}CD11b^{+} cDCs performed this role in the large intestine [190]. These studies suggest that IRF4 and KLF4 dependent cDC2s are required for the development of Th2 immunity, while the mechanisms behind this remain to be elucidated. Infection with *Trichuris muris* in CD11c-cre.*Irf4*$^{fl/fl}$ leads to reduced expression of Th2 related cytokines such as IL-13, IL-4 and IL-5, which may lead in combination with impaired function of MHCII antigen processing to reduced Th2 responses [186, 189]. In contrast to mice with CD11c specific KLF4 deletion, Notch2 deficient mice can drive normal Th2 responses but are impaired in driving Th17 responses [179], indicating a functional heterogeneity in cDC2.

Finally, although cDC1 were superior in inducing pTreg in response to dietary antigen, the same study by *Esterhazy et al.* showed that CD103^{+}CD11b^{+} cDC2s isolated from MLNs could induce differentiation of OT-II cells into pTregs in vitro [167]. Moreover, *huLangerin*-DTA x BATF3$^{-/-}$ mice that lack both CD103^{+}CD11b^{-} cDC1 and CD103^{+}CD11b^{+} cDC2s have reduced (CCR9^{+}) Treg numbers in the small intestine but not the MLN [188], indicating that both subsets, which are mutually redundant for Treg induction, are jointly required for imprinting of a gut homing signature in Tregs. Thus the intestinal mucosa contains several cDC subsets, that appear to play distinct and possibly complementary roles in intestinal adaptive immune homeostasis.

Chapter 4 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is the collective name for different chronic inflammatory disorders of the intestine. Crohn's disease (CD) and ulcerative colitis (UC) are the main types of IBD. Both CD and UC lead to long-term and sometimes irreversible damage to the intestinal epithelium [192], however they target different regions of the intestinal tract, and have distinct pathology. CD can affect any part of the gastrointestinal tract, although it is commonly concentrated to the terminal ileum. In CD transmural inflammation is more common, in combination with a thickened mucosa where aggregation of macrophages form granulomas [193, 194]. In contrast, UC is mostly restricted to the distal colon [192, 195], with a more superficial inflammation that is limited to the mucosa and submucosa, and can lead to cryptitis (inflammation of the crypts) and crypt abscess formation through accumulation of neutrophils in the lamina propria. The symptoms associated with IBD include diarrhea, fatigue and weight loss; furthermore, while abdominal pain is more common in CD, bloody stools predominantly occur in UC patients [196, 197]. Western Europe, North America and Australia have the highest reported prevalence of IBD worldwide [198]. However, prevalence of IBD is increasing worldwide, even in areas previously considered as low-prevalence regions, e.g. Asia, the Middle East and South America [196-198].

4.1 Risk factors

Several risk factors have been associated with IBD, including genetic predisposition and environmental and dietary factors. Genome-wide association studies (GWAS) have identified numerous risk loci for IBD. These include genes associated with intestinal barrier function, e.g. genes involved in autophagy and paneth cell function or tight junction formation [194, 199], as well as immune-related genes associated with innate or adaptive immunity, e.g. the recently identified polymorphisms in DC-related genome regions (e.g. DEC-205 and CCL20) [199, 200], or genes involved in Th17 functionality or modulation of general components of T cell activation (e.g. AHR and CD28) [201]. These risk loci will be discussed in more detail in chapter 4.3 (*'Innate immune system in IBD'*).

Certain gene loci are a significant contributing factor for IBD pathogenesis such as NOD2, people carrying one high risk allele had 2.39 fold increased odds to develop CD and people carrying two copies of a high risk allele had an 17.7 fold increased risk for developing CD compared to people that carried no copies of high risk alleles [202]. Of all identified risk loci NOD2 has the highest contributing factor [202]. However, the fact that IBD is more common in certain geographical regions and that it increases rapidly in 'genetically stable' populations indicates that environmental factors have a major contribution. For example, epidemiological studies have demonstrated that IBD is more common in westernized regions with a lower prevalence in rural compared to urban areas [198, 203]. Furthermore, young immigrant populations migrating from low- to high-prevalence regions were shown to adopt a similar incidence rate compared to non-immigrant populations in the high prevalence regions [204].

Increased allergies and autoimmune diseases such as IBD in the industrialized world has been associated with a decline in (childhood) infections, also known as the hygiene hypothesis [205, 206]. Consistent with this idea, childhood exposure to pets or living on a farm in combination with drinking unpasteurized milk and higher housing density have been correlated with reduced risk for developing IBD [207]. Although there is little doubt that environmental factors can contribute to the risk of developing IBD, many of the low prevalent regions are industrializing, this often is connected to improvement of healthcare and in combination with better knowledge about IBD diagnostics this might increase the amount of reported cases in certain regions.

Dietary factors have however also been identified; increased risk for IBD was found to correlate with a high intake of sugar/sweeteners and fat-rich diets, while breast-feeding and high intake of dietary fiber was correlated with reduced risk [208, 209]. The majority of these studies indicate dietary and other exposing factors that may influence the commensal microbiota, which is consistent with the emerging idea that cross-talk between the microbiota and the immune system is essential for maintaining immune homeostasis in the intestine [3, 210]. Exposure to antibiotics, especially in childhood, is hypothesized to alter development of tolerance to enteric bacteria, which may lead to IBD, while other studies have identified the use of antibiotics years prior

to IBD development as an increased risk [208, 209, 211]. Furthermore, decreased biodiversity and dysbiosis have been indicated as one of the driving forces in IBD [212]. Although some pathogens have been associated with increased risk (e.g. increase in adherent-adhesive *Enterobacteriaceae*), there are also members of the microbiome that have protective effects in IBD by for example down-regulation of inflammatory cytokines (e.g. *Bifidobacterium, lactobacillus, faecalibacterium*) [212, 213]. Collectively, the above-mentioned studies show that environmental and dietary factors influence the intestinal immune system either directly or indirectly via the microbiota and this may lead to IBD in genetically susceptible individuals (*see figure 1. for a schematic overview*). Moreover, in multiple mouse models of colitis such as Dextran sulfate sodium (DSS) induced colitis and T cell transfer colitis, germ-free mice develop no or only mild colitis [214, 215]. It is so far not known if colitis in IBD patients develops due to dysbiosis or if dysbiosis is a consequence of colitis, this may even differ between patients.

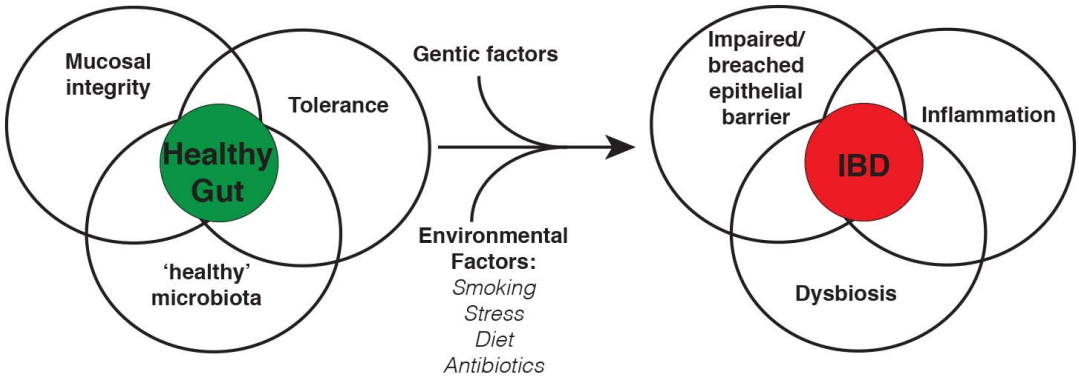

Figure 1. Schematic overview of factors that can lead to IBD. Genetic and environmental factors may lead to impaired intestinal barrier function, dysbiosis and inflammation, which together may result in IBD.

4.2 Animal models for IBD

Although animal models for IBD do not represent the complexity of the human disease, they have contributed greatly to our understanding of the underlying immunological mechanisms that drive and maintain chronic intestinal disease. This chapter will describe a selection of the most commonly used murine models of IBD including the T-cell transfer colitis model that was used for manuscript I.

Chemically induced colitis: Chemically induced models are the most frequently used; they are simple and the onset of inflammation is rapid. Administration of DSS in drinking water results in symptomatic features that resemble UC [216]. DSS damages the epithelial barrier resulting in increased intestinal permeability, bacterial translocation and acute inflammation. Acute colitis is induced by one continuous cycle of DSS (4-9 days) and chronic colitis follows after administering low doses of DSS in consecutive cycles (7 days followed by water for 10 days) [217]. Colitis is accompanied by increased expression of inflammatory mediators (e.g. TNFα, IL-1β and IL-6, IFNγ), increased epithelial apoptosis, tight junction alterations and increased infiltration of inflammatory cells. Trinitobenzene sulphonic acid (TNBS) is injected rectally and like DSS disrupts the mucosal barrier, inflammation is induced by haptenization of colonic mucosal proteins [218]. TNBS colitis is characterized by Th1 driven inflammation [219]. Rectal administration of Oxazolone induces symptoms earlier in comparison to TNBS and is characterized by ulceration in the distal colon, resembling UC [220].

Innate anti-CD40 model of colitis: The model of anti-CD40 induced colitis in *Rag*$^{-/-}$ mice is particularly suitable to study innate immunity in colitis. Injection of agonist anti-CD40 monoclonal antibody, which resembles the CD40L on activated T cells, in Rag$^{-/-}$ mice results in acute innate driven colitis [221].

Genetic models of colitis: IL-10 deficient mice develop spontaneous microbial induced colitis [222, 223], which is predominantly mediated by aberrant Th1 immune responses [224]. IL-10 is an important anti-inflammatory cytokine that directly inhibits macrophage and Th1 cell function [223]. TCRα deficient mice develop microbial driven colitis [225, 226] and a strong antibody response to self antigens [226]. Disease in TCRα deficient mice is characterized by type 2 immunity [224].

Adoptive transfer model: The model of CD4^{+}CD45RB^{hi} T cell transfer is probably the most suitable model to study CD4^{+} T cell driven colitis. Transfer of naïve CD45RB^{hi} T cells into Rag$^{-/-}$ or severe combined immune deficient (*scid*) recipients results in severe chronic colitis, the transferred cells polarize and expand into Th1 and Th17 cells upon interaction with microbiota derived antigen [227]. This model has been instrumental in identifying the T cell subsets that are driving IBD and the effector

cytokines driving and controlling this response. One of the key findings in the T cell transfer model of colitis is that colitis can be prevented or cured by co-transfer with $CD4^+CD25^+CD45RB^{lo}$ regulatory T cells [118]. Thus this model is also of use for understanding the mechanism of T cell mediated control of colitis.

Pathogen induced models of colits: *Rag*$^{-/-}$ or *scid* mice adoptively transferred with $CD4^+CD45RB^{hi}$ T cells or IL-$10^{-/-}$ mice that are infected with *Helicobacter hepaticus* develop severe colitis that is often accompanied with rectal prolapse [228]. Chronic intestinal inflammation in Helicobacter hepaticus infected colitic mice is predominantly Th1 driven, with elevated levels of TNFα, IFNγ and IL-12 [229]. *Citrobacter rodentium* is a mouse pathogen similar to entero-pathogenic *Escherichia coli* in humans [230]. *C.rodentium* that infects the ceacum in mice induces a strong mucosal Th1 response that causes pathology similar to Th1 driven IBD in humans [230].

4.3 Genetics and immunology of IBD

As introduced in chapter 1, the intestinal tract is continually exposed to a large amount of foreign antigen such as food antigens, pathogens and commensal microbiota. The complex task of the intestinal immune system is to distinguish the beneficial antigens from the detrimental ones; failure in this balancing act, in combination with environmental factors and genetic predisposition, can result in inflammatory responses that may develop into chronic intestinal inflammation, including IBD. Several direct or indirect immune-related functions and pathways have been revealed by GWAS as risk loci for IBD [231]. This chapter will describe the main genetic risk factors and consequences of these mutations that are connected to the development and maintenance of IBD.

4.3.1 innate immune system in IBD

Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) (a.k.a. CARD15) was one of the first identified risk genes for IBD. Polymorphisms in this gene are common among IBD patients and have been associated with direct and indirect effects on innate immunity [199, 232]. NOD2 is a member of the PRR family that recognizes muramyl dipeptide (MDP), a component of bacterial peptidoglycan

[233]. NOD2 is broadly expressed by macrophages, dendritic cells and at lower levels in epithelial cells [233]. Activation of NOD2 by MDP results in TRAF6-mediated activation of the NF-κB pathway, leading to secretion of IL-1 and IL-8 [233, 234]. Most variants of NOD2 polymorphisms interfere with the ability of NOD2 to recognize its ligand [235], resulting in reduced capacity to induce NF-κB activation and IL-1 production upon stimulation with MDP [235]. Indeed cDCs derived from CD patients with NOD2 deficiencies have an impaired ability to produce IL-1α/β in co-culture with memory T cells [236]. Although it is indicated that NOD2 mutations can contribute to colitis in a large group of IBD patients [231], it remains to be elucidated whether direct effects on NOD2 signaling by cDCs contributes to disease development. NOD2 has also been suggested to regulate responses via other PRRs such as downregulation of IL-12 production via TLR2 [235]. Indeed, the inability of NOD2 variants to downregulate TLR2-induced IL-12 has been suggested as a major contributor to aberrant the inflammatory response in CD [235]. Moreover in a model of necrotizing enterocolitis, activation of NOD2 inhibited TLR4-signaling and thereby ameliorated disease [237]. In the healthy murine and human colon DCs express low levels of TLRs and DC activation markers such as CD80 and CD86 [238-240]. This is likely contributing to their relatively quiescent phenotype in homeostatic conditions [239, 241]. cDCs from IBD patients however have an activated phenotype, indicated by significantly elevated expression of TLR2, TLR4 [239]. Moreover, especially cDCs from inflamed tissues of IBD patients had increased expression of CD40 and showed enhanced production of IL-12, IL-6 compared to those from un-inflamed tissues or healthy controls [239]. Whether this increased activity in cDC from IBD patients is related to NOD2 mutations has however not been assessed.

More recently it was demonstrated that NOD2 signaling can induce autophagy ('self-eating') at sites of bacterial entry, via ATG16L1 [242, 243]. Autophagy is another important cellular pathway that has not been explored in relation to IBD until relatively recently [244]. Autophagy occurs essentially in all cells of the body as a homeostatic function to regulate organelle turnover, but is upregulated in the event of starvation to generate intracellular nutrients and energy, and in order to remove cells that are damaged following intracellular infection, protein aggregate formation, accumulation of misfolded proteins, or oxidative stress [245]. Monocyte derived DCs from IBD patients with NOD2 polymorphisms have reduced autophagy and impaired

MHCII surface expression upon stimulation with MDP that resulted in diminished bacterial killing of adherent-invasive *E.coli* by DCs with NOD2 polymorphisms in *vitro* [243].

Both NOD2 and ATG16L1 have also been implicated in IL-10 signaling pathways [246]. A gain-of-function variant of NOD2 polymorphism has been suggested to suppress IL-10 transcription [247] and monocytes from IBD patients with NOD2 and ATG16L1 polymorphisms had significantly impaired IL-10 production upon *in vitro*-stimulation [246]. Moreover, children with functional mutations in IL-10 receptor (IL-10R) display severe IBD-like symptoms in the first years of their lives [248] and spontaneous microbiota-driven colitis also arises in IL-$10^{-/-}$ mice [23]. IL-10 is an important mediator for $FoxP3^{+}$ Treg survival and it suppresses IL-23p19 and IL-12p35 [22, 23, 143]. Furthermore, IL-10R on macrophages is essential for intestinal homeostasis as mice lacking IL-10R on macrophages develop spontaneous colitis [30, 31]. Although these studies indicate important functions for NOD2 signaling and autophagy in regulating intestinal homeostasis, it remains to be determined whether polymorphisms in these genes directly lead to IBD. It is for example still unknown which cell types promote colitis in patients with NOD2 mutations.

Ly75 that encodes for the endocytotic c-type lectin receptor 205 (DEC-205), a surface receptor that is highly expressed on DCs, is involved in the endocytosis of exogenous antigens and their presentation on MHC, is a relatively newly identified risk locus for IBD [199]. Although the mechanism in IBD patients with *Ly75* polymorphisms remain to be elucidated, in experimental colitis targeting of DEC-205 that is expressed on immature DCs promotes $FoxP3^{+}$ Treg proliferation [249, 250]. Targeting of DEC-205 leads to ameliorated disease in a model of villin specific $CD4^{+}FoxP3^{-}$ Tcell induced colitis [249]. Another recently identified loci is *CCL20* it encodes for the chemokine CCL20 that is expressed by intestinal epithelium and is involved in the recruitment of regulatory T cells and dendritic cell to the gut [251]. This chemokine is increased in the intestine of IBD patients [251, 252]. Moreover, the locus of its receptor CCR6 has also been associated with IBD [199]. The mechanisms by which CCL20 and CCR6 contribute to IBD remain to be elucidated, but in inflamed crypts of IBD patients it was shown that increase in $CCR6^{+}$ DCs was positively correlated with a higher histological inflammatory grade [253]. CCR6 is

also involved in the homing of Th17 and Treg cells to the intestine [254], and interestingly transfer of Th17 cells from $CCR6^{-/-}$ donors resulted in severe Th1 mediated colitis in *Scid* mice [254]. Interestingly, $CCR6^{-/-}$ mice were partially protected from DSS induced colitis [255].

Collectively these studies indicate that the innate immune system has a significant role in the onset and maintenance of IBD. Innate immune cells can create a pro-inflammatory environment by secreting cytokines but they can also act as a mediator to attract other cells of the innate and adaptive immune system. (*See figure 2. for an overview of immunological changes in IBD*)

4.3.2 Adaptive immunity in IBD

Key insights from GWAS has implicated a role for the IL-23/Th17-axis in IBD [231, 256]. For a long time, under the influence of the prevailing Th1/Th2 paradigm, UC was long considered a Th2-driven disease whereas Th1-immunity was suggested as a main driver of CD [193, 257-260]. However, with the discovery of Th17 cells [92], and that IL-12 shares the p40 subunit with IL-23, this picture has changed [261]. Furthermore, additional genes involved in Th17 differentiation have been revealed as IBD-associated loci [231, 262] and polymorphisms in the IL-23 receptor gene that result in enhanced signaling are highly correlated with an increased risk for IBD [194, 231, 263]. As mentioned in chapter 2.4, IL-23 is not involved in the differentiation of Th17 cells but it is important for their maintenance and (pathogenic) effector functions [95]. In recent years the IL23/Th17 axis has been implicated in many immunopathological disorders including IBD [264, 265], and below is a summary of our current understanding of Th17 cells and IL-23 in intestinal pathology.

Mucosal inflammation is characterized by the recruitment of monocytes from the circulation in response to upregulation of inflammatory cytokines and chemo-attractants such as CCL2 [266, 267]. These monocytes can give rise to inflammatory macrophages and monocyte derived DCs (moDCs), which are both potent inducers of TNF, IL-12 and IL-23 [142, 143, 193, 268]. These moDCs and monocyte derived macrophages both in humans and mice are the main producers of IL-23 and in mice $CD103^{+}CD11b^{+}$ intestinal cDCs have also been shown to express IL-23 [143, 184, 268, 269]. IL-23 binds to the IL-23R on Th17 cells and both in humans and mice

stimulates the production of IL-17, IL-21 and IL-22 and IL-6 [270, 271]. The IL-17R is widely expressed by immune cells but also by non-hematopoietic cells such as endothelial- and epithelial cells [272, 273]. Binding of IL-17 to the IL17R promotes pro-inflammatory cytokine production by intestinal epithelial cells such as TNF, IL1 and IL-6 [273-275].

The importance of IL-23 in intestinal inflammation has been confirmed by many studies in mouse models for experimental colitis. Spontaneous colitis in IL-$10^{-/-}$ mice was abrogated in the absence of IL-23p19, but not in the absence of IL-12p40 [276]. Colitis was also abrogated in the absence of IL-23p19 in a model of T cell-transfer colitis and *Helicobacter hepaticus*-infected mice treated with anti-IL-10R [277]. Initial experiments indicated that the pro-inflammatory actions of IL-23 was primarily mediated through its activities on Th17 cells, however more recently ILCs and unconventional γδT cells were also found to be responsive to IL-23 [278, 279]. In IBD patients RORγt^{+} ILCs that responded to IL-23 are a source of IL-17 in inflamed tissue [280] and γδT cells express Th17 related transcription factors such as RORγt and AHR, and can secrete IL-17 and IL-22 in the response to IL-23 and IL-1β [278, 279, 281]. The involvement of innate immunity in IL-23 dependent colitis was shown in *Helicobacter hepaticus*-driven colitis in *Rag*$^{-/-}$ mice where colitis was abrogated in the absence of IL-23 [282]. The involvement of IL-23 in innate immune-driven colitis was later confirmed in the anti-CD40 model of colitis in *Rag*$^{-/-}$ mice [283], and it was suggested that RORγt^{+} ILCs drive IL-23 dependent intestinal pathology in the anti-CD40 model of colitis [283]. These findings of ILC involvement in mouse models of colitis are however not yet confirmed in human disease.

Group 1 T-bet expressing ILCs, that could produce IFNγ ex vivo, are present in higher numbers in inflamed intestinal tissues from CD patients [284]. Interestingly these IFNγ expressing ILC1s could develop from group 3 RORγt^{+} ILCs, indicating that there is a certain plasticity between the group 1 and group 3 ILCs [284]. Similar events have been identified in Th17 cells; especially in inflammatory conditions Th17 cells can upregulate IFNγ under the control of IL-12 and IL-23 but in the absence of TGFβ [285-287]. Moreover, an increase in IL17^{+}IFNγ^{+} T cells has been observed in patients with active IBD [288].

More recent studies indicated that IL-23 may not only act as a mediator of pro-inflammatory Th17 cells, but can also act to reduce the amount of FoxP3$^+$ Tregs in T cell-transfer colitis [289]. Transfer of naïve T cells in IL-23 deficient *Rag*$^{-/-}$ mice resulted in a significantly increased proportion of FoxP3$^+$ T cells compared to transfer in IL-23 sufficient *Rag*$^{-/-}$ mice [289]. This indicates that apart from maintaining colitis-inducing Th17 cells, IL-23 may also act to suppress regulatory responses. Th17 and FoxP3$^+$ Tregs are developmentally related as TGFβ is required for the differentiation of both populations, and studies have indicated that the presence of STAT3-mediated signals, such as IL-6 and IL-23 promote Th17 cells at the expense of FoxP3$^+$ Tregs [290, 291]. Furthermore, TGFβ-signaling is impaired in the inflamed intestine of IBD patients [292]. SMAD family member 7 (Smad7) associates with TGFβ and functions as an antagonist for TGFβ signaling [293]. The impaired TGFβ signaling was correlated with an increased expression of Smad7 [292, 294]. Blockade of Smad7 was shown to restore TGFβ-signaling in a patients with active IBD and although the effect on disease severity was not measured, mononuclear cells isolated from these patients showed decreased pro-inflammatory cytokine production after Smad7 treatment [292].

The notion that IL-23 is the main driver of intestinal pathology might be too simplified, as interplay of IL-23 and IL-12 has been shown to maintain chronic local and systemic inflammation in IBD and other chronic inflammatory diseases [221, 295-297], and in CD patients both IL-23 and IL-12 are elevated [298, 299]. Thus, it has been suggested that IL-23 regulates local bacterial-induced inflammation in the colon, whereas IL-12 directs innate driven systemic inflammation [221]. Other cytokines may be involved as well, such as the more recent identified IL-21. IL-21 is increased in the inflamed intestine of IBD patients and is produced by both Th17 and Th1 cells [193, 194, 300]. Blocking of IL-21 suppressed the production of IFNγ and IL-17 by in *vitro* stimulated T cells isolated from IBD patients [300-302]. Moreover IL-21 deficient mice were protected from DSS- and trinitrobenzene sulfonic acid (TNBS)-induced colitis [302]. The pro-inflammatory effect of IL-21 may be enhanced by its ability to suppress Treg responses, as CD4$^+$CD25$^+$ Tregs treated with IL-21 failed to prevent T cell-transfer colitis in *Scid* mice [303]. All these data together indicate that Th17 derived cytokines could have both detrimental and protective

effects, IL-23 seems to play a role in the regulation and maintenance of pro-inflammatory effector roles of Th17 cells in IBD [256].

Although in some patients a single gene defect can lead to severe early onset IBD, such as mutations in the IL-10 gene, in most cases IBD develops due to a combination of genetic pre-disposition, environmental influences and a slow disease development due to a change in barrier function, cell infiltration and function, and the cytokine environment. Together the studies mentioned above illustrate the immune complexity of IBD. (*See figure 2. for an overview of immunological changes in IBD*)

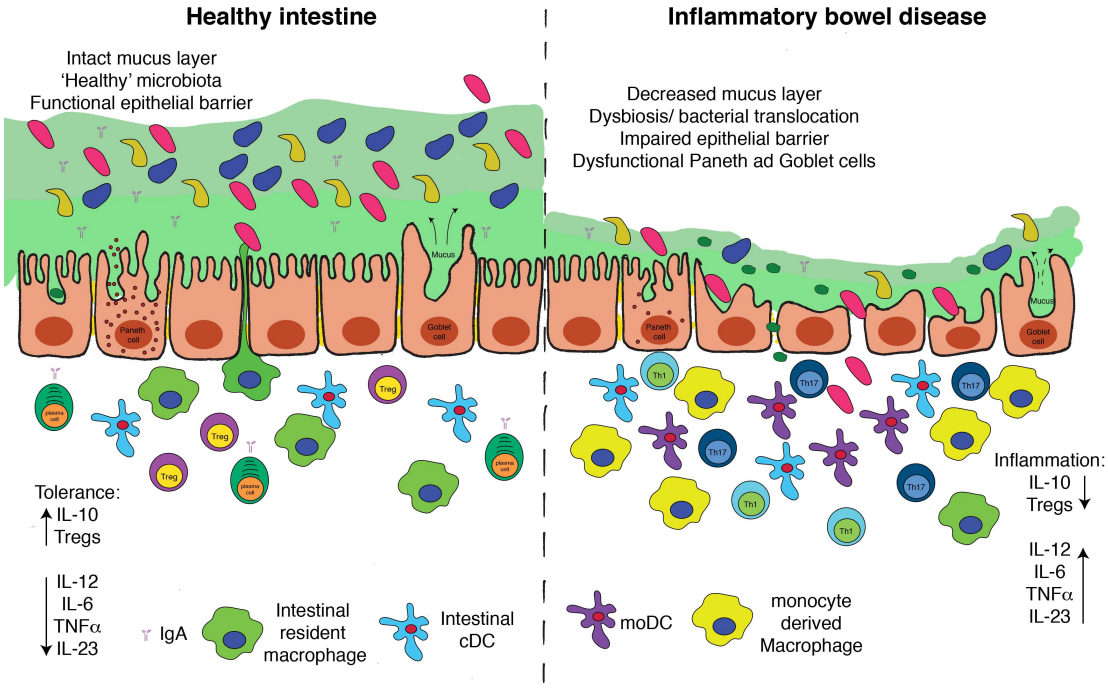

Figure 2. An overview of the biological and immunological changes in IBD. In IBD, intestinal integrity is impaired through damage of the epithelium, and impaired function of paneth cells (less antimicrobial peptides) and goblet cells (impaired mucus production). Impaired barrier integrity results in dysbiosis and increased bacterial translocation. Classical dendritic cells (cDCs), macrophages and epithelial cells respond to the breach of the epithelium by producing pro-inflammatory cytokines and chemokines. cDCs upon capturing intestinal (bacterial) antigen travel to the MLN to prime and imprint Th1 and Th17 cells with a gut homing signature. Increased levels of pro-inflammatory cytokines and chemokines attract monocytes from the circulation that develop into monocyte derived DCs (moDCs) and monocyte derived macrophages. moDCs and monocyte derived macrophages produce high levels of IL-23 that induces a pro-inflammatory Th17 response and inhibits Tregs function.

4.4 Treatments for IBD

There is no known cure for IBD, but anti-inflammatory drugs are frequently used in the treatment for IBD to help reduce symptoms and retain remission. The choice of

treatment is usually based on the current disease activity, severity and long-term prognosis in combination with negative responses or side effects in prior treatments [196, 197]. Mild disease is often treated with aminosalicylates and corticosteroids are prescribed for patients with moderate disease. Severe UC is often treated with cyclosporine and current treatment for severe fistulizing CD are biological therapeutics (e.g. anti-TNFα) [304]. Anti-TNF agents have been a major advancement in the treatment of severe IBD of which infliximab and adalimumab are the most frequently used and these are mostly the first biological treatment to be used in patients [305, 306]. Anti-TNFα antibodies neutralize soluble TNFα, induce apoptosis of- and block growth factors for activated T effector cells and inflammatory monocyte derived cells and they can induce regulatory macrophages in the local tissue [307, 308]. Although anti-TNFα antibodies have proven to be effective and reasonably safe, despite earlier concerns [309], about one third of individuals are unresponsive to this treatment apart from the fact that it is relatively costly [305]. In long-term treatments patients can develop immunogenicity by generating anti drug antibodies, this could be overcome by administering immunosuppressive drugs simultaneously, even though it can lead to increased risk for infection and malignancy [304, 310, 311].

Other biological agents that are investigated for IBD treatment are anti adhesion molecules (anti-α4β7), JAK inhibitors, anti IL-12/IL-23p40 antibodies and restoration of normal TGFβ signaling (SMAD7 blockage) [305, 312, 313]. Surprisingly despite pre-clinical indications that IFNγ and IL-17 play major roles in the maintenance of IBD, clinical trials were unsuccessful. Anti-IFNγ failed in clinical trails and anti-IL-17 even leads to worse disease in CD [314, 315].

Microbiome modulators such as antibiotics, prebiotics (e.g. carbohydrates, short-chain fatty acids), probiotics, special dietary formulations and fecal microbial transplantation are under investigation. Some trials have shown minor success but long-term effects of dramatic changes in the microbiota are unknown and large scale studies are needed to get a clear picture of what a healthy microbiome actually is [305, 316, 317].

Chapter 5 Colitis associated cancer

IBD patients are at increased risk for developing colorectal cancer (CRC), either in sporadic form or as colitis associated CRC (CAC) [318-320]. CRC is the third most common cancer worldwide, and accounts for about 8% of cancer related deaths [320]. The risk for CRC in UC patients is 2% after 10 years, 8% after 20 years and 18% after 30 years of active disease [321]. Later studies however found a decreasing risk of CRC in IBD patients [321, 322], which might be related with the improved treatments of IBD and better surveillance for dysplasia [323]. This risk for CRC in IBD patients is correlated with the severity of colitis, local inflammation and family history; IBD patients with primary sclerosing cholangitis and/or a family history of CRC are at high risk but patients without colonic inflammation and UC limited to the rectum have no increased risk for developing CRC [322, 324].

5.1 Risk factors for colorectal cancer

Although genetic factors have been implicated in sporadic CRC (e.g. mutations in the tumor suppressor *APC* gene), they are less clear defined in CAC where mutations mostly occur due to excess inflammation [325]. However approximately 5-10% of colon cancers are initiated by inheritable mutations [326]. CRC has like IBD been associated with a 'western' lifestyle, high consumption of animal fat, processed or red meat, low intake of vitamin D and a diet poor in fibre and fish have been linked to risk of CRC development [327, 328]. Some studies have reported association of dysbiois and the development of CRC [329] and some bacterial populations have been identified to be present in higher number in the CRC affected colon (e.g. *fusobacterium*) [330, 331]. The risk factors that contribute to CAC, are less well studied compared to sporadic CRC, as formation of cancerous lesions are more likely to result from chronic inflammation of the mucosa then from any clear-cut genetic predisposition.

5.2 Animal models for CRC and CAC

Genetic models: Mice carrying a heterozygous mutation in the APC gene rapidly develop tumors similar to patients with familial adenomatous polyposis. This model has been very helpful in understanding the polyp growth and progression and in the functional analysis of the APC gene product and mapping of the essential domains

[332]. About 60% of IL-$10^{-/-}$ mice develop tumors in the proximal colon and ceacum, these tumors do however not resemble human CRC or CAC, no alterations in the p53 or APC gene or mismatch repair defects were found in these mice [333].

Chemically induced models: Carcinogen induced models in mice are a valuable tool to assess phases of initiation and progression of tumors that occur in human CRC. Chemical induced models are relatively reproducible and can be applied to animals with different genetic backgrounds. The currently most common carcinogen used is Azoxymethane (AOM) [334]. Upon intra-peritoneal injection, AOM gets hydroxylated and forms the reactive metabolite MAM that can alkylate macromolecules in the liver and colon [335]. This process leads to methylation at the O6-position of guanine in the DNA molecule, which has been shown to be the primary pro-mutagenic lesion produced by AOM [335]. AOM induced tumors carry frequent mutations in K-Ras and β-catenin and less common microsatellite instability [334]. Administration of several cycles of DSS in combination with AOM dramatically decreases the latency time, causing rapid tumor growth within 10 weeks compared to 30 weeks with AOM administration alone [336]. In the AOM/DSS induced model of CAC tumors develop from aberrant crypt foci that develop into adenomas at about week 5-6 that form into carcinomas at about week 8-20 depending on the mouse strain [334].

Pathogen induced models: IL-10 deficient mice that are infected with *H.hepaticus* develop tumors more rapidly compared to uninfected mice with IL-10 deficiency [337]. Tumors that formed in this model were comparable to AOM/DSS induced tumors in terms of morphology, invasiveness, or β-catenin mutations [337].

5.3 Immunopathology of colitis associated cancer

The inflamed colon in IBD patients is rich in reactive oxygen species (ROS) due to the infiltration of among others inflammatory macrophages which can cause DNA damage and exogenous mutations (e.g. microsattelite instability, CpG island methylation and microRNA alterations) facilitating the initiation of cancerous lesions [318, 338]. Cumulative effects of DNA damage and p53 (tumor suppressor) mutations results in continuous activation of Wnt/β-catenin signaling pathway, and formation of

adenomatous lesions in the colon, eventually resulting in the loss of the adenomatous polyposis coli (*APC*) tumor suppressor gene function [326]. P53 mutations occur in a later stage in large adenomas of sporadic CRC patients while in contrast in CAC cytokine stimulation drives the p53 mutation in inflamed mucosa in the early stage often before dysplasia occurs [323, 326] (*see figure 3. for a schematic comparison of CRC and CAC)*.

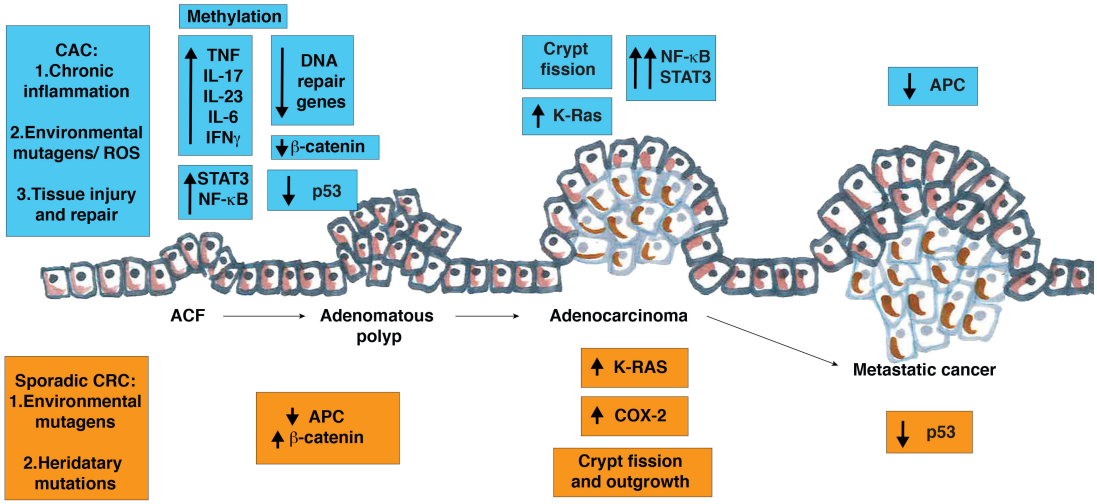

Figure 3. Mechanism of colitis associated colorectal cancer (CAC) and sporadic colorectal cancer (CRC) development. CAC follows upon increased levels of pro-inflammatory cytokines, and increased STAT3 and NF-κB signaling. This can induce mutations in oncogenes such as *APC* and *K-Ras* and genomic instability by for example methylation as seen in AOM/DSS induced colitis. Persistent inflammation facilitates tumor promotion by activating proliferation and anti-apoptotic properties of premalignant cells, as well as tumor progression and metastasis. CRC in contrast is caused by accumulation of mutations in oncogenes and tumor suppressor genes (e.g. APC) or aberrant activation of β-catenin. Mutations in APC or β-catenin, or other components in this pathway mediate the transition of single neoplastic cells to aberrant crypt foci (AFC) and then to adenocarcinoma and metastatic cancer.

Impaired barrier integrity and epithelial cell damage due to chronic intestinal inflammation results in increased epithelial turnover and bacterial translocation. The fact that germ free mice have reduced tumor development [339] and that MyD88 deficient mice have reduced tumor numbers indicates that PRR signaling is an important factor in CAC development [340]. PRR signaling initiates NF-κB mediated IL-6 production in myeloid cells that has been thought to activate STAT3, which is an important regulator of proliferation and survival of tumor-initiating intestinal epithelial cells (IEC) both in AOM/DSS induced CRC [341-345]. Moreover, STAT3 activation by IL-6 (or alternatively by IL-22 and IL-23) has been implicated to regulate the tumor inflammatory environment directly by inducing pro-tumor gene

expression programs such as the expression of angiogenic factors [341, 346, 347]. Consistent with this, epithelial cells from patients with active UC and patients that had progressed to CAC, expressed higher levels of IL-6 and phosphorylated STAT3 protein expression [348]. In contrast, stimulation of STAT1 by type 1 interferons regulates expression of genes that inhibit growth and induce apoptosis of malignant cells [349]. The above studies indicate that signaling via STAT proteins shows to be a double-edged sword in tumor immunity, where signals such as IFNγ, IL-12, IFNα/β and IL-18 promote tumor elimination via STAT1 while IL-6, IL-22, indoleamine 2,3-dioxygenase (IDO), IL-10 and TGFβ promote tumor escape via STAT3 [341, 349-352].

TNFα, as described in the previous chapter, is an important cytokine in IBD pathogenesis as treatment with anti-TNFα MAb shows to be successful in a majority of patients. Classically considered an anti-tumor agent, it has now also been indicated to promote tumor growth during chronic inflammation. TNFα can promote angiogenesis and induce expression of cyclooxygenase-2 (COX-2) an enzyme that by itself can promote angiogenesis as well [353]. Moreover, TNF-R deficient mice treated with AOM/DSS had reduced inflammation and tumor formation compared to wild type controls [354]. Furthermore, *Rag*$^{-/-}$ mice with *H.hepaticus* infection develop CAC, but this was suppressed by the neutralization of TNFα [355]. Neutralization of TNFα or TNF-R deficiency foremost leads to reduced inflammation in the above mentioned models, this gives rise to the question if the reduced tumor burden is a result of the direct effect of TNFα on tumor formation or if a less inflammatory environment is less pro-tumorigenic.

Lower levels of Th1 and cytotoxic T cell related mRNA, such as T-bet, IFNγ and granzyme, were measured in tumors of CRC patients with early metastatic invasion compared to tumors from patients without such signs [356]. This and other studies indicated that increased Th1 and cytotoxic T cell infiltration in the tumors correlated with increased patient survival [356-358].

Finally, removal of immune suppressive signals such as TGFβ results in increased intra-tumoral IFNγ, Th1 and cytotoxic $CD8^+$ cells and reduced tumor burden [359]. Thus, tumor induced immune-evasion in human CRC and murine AOM/DSS induced

CAC is partly mediated by enhanced expression of immunosuppressive cytokines such as TGFβ and IL-10 within the tumor environment [313, 350]. Moreover IDO expression by antigen presenting cells has been implicated in the activation of Tregs in AOM/DSS induced CAC [351], and enhanced IDO expression in human CRC is correlated with worse prognosis [360].

Clinical and experimental data indicate that chronic intestinal inflammation in CAC act as a double-edged sword. Release of cytokines such as IL-6 and TNFα during chronic colitis can promote tumor growth and regulatory factors such as TGFβ can exacerbate this process. Thus, several factors that have been shown to be protective in IBD, such as IL-22, IL-10 and TGFβ have been indicated as important tumor promoting factors in CAC [347, 359, 361]. While for example Smad7 was shown to inhibit TGFβ-signaling in IBD patients which is thought to promote colitis [292], in another study the Smad7 expression in colonic tissue of IBD patients was correlated with less IBD related complications such as CAC [359]. Similar findings have been published for IL-22, IL-22 administration ameliorated experimental colitis and IL-22 induced STAT3 signaling has been indicated to be important in mucosal wound healing in DSS induced colitis [362, 363]. While both in human CRC and experimental CAC IL-22 has been indicated to induce tumors in a STAT3 dependent manner [352, 364].

Collectively data from these studies indicate that treatment of IBD and thereby reducing the inflammation in the intestine decreases the risk or CAC, but that patients with progressed disease and signs of dysplasia may require different treatment. It is currently unknown what impact most anti-inflammatory drugs and immune modulators have on IBD-related CAC, and if any of these drugs improve or dampen the immunosurveillance against dysplastic cells. Although clinical data indicate that 5-aminosalicylate (5-ASA) may prevent CAC in UC patients [365].

5.4 Prognosis, surveillance and treatment of CRC/CAC

Survival rate of CRC patients is about 50% in the first 5 years after diagnosis, with only 6 months average survival in untreated metastatic CRC [366]. To decrease the CAC cancer complications in IBD patients it is becoming common practice to

perform regular surveillance colonoscopy, however dysplasia is very difficult to distinguish from inflamed tissue, targeted biopsies are therefore recommended to be taken during surveillance [367]. Chemo-preventive agents can inhibit, delay or reverse colon carcinogenesis, 5-aminosalicylic acid (5-ASA) is an attractive candidate due to its indicated anti-carcinogenic effect, however clinical data is conflicting and needs further investigation [368]. Increasing evidence is indicates that non-steroid anti-inflammatory drugs (NSAIDs) can prevent premalignant polyps, CRC onset and recurrence [366]. In established CRC cytotoxic chemotherapy (e.g. 5-fluorouracil) in combination with biologicals (e.g. anti-endothelial growth factor (VEGF)) are frequently used [366].

Aims of the thesis

The overall aim of this thesis work was to study the role of intestinal dendritic subsets in experimental models of T cell induced colitis and AOM/DSS induced CAC.

More specifically:

1. What is the role of IRF4 expressing cDC2s in T cell induced experimental colitis.
2. What is the role of IRF8 expressing cDC1s in AOM/DSS induced CAC

Concluding remarks and future perspectives

This thesis work summarizes current relevant knowledge on the intestinal immune system and the immunological alterations that contribute to pathologies of the intestinal tract. Our understanding of cDC lineages and their development and functionality has evolved significantly over the last decades. Their involvement in inflammatory bowel disorders and associated colorectal cancer however has not been studied in detail.

Manuscript I describes that IRF4 expressing cDC2s have a role in the initial priming of colitogenic T cells and priming and maintenance of Th17 cells during colitis. Absence of IRF4 expressing cDC2s resulted in delayed onset of colitis. Absence of IRF4 expressing cDC2s interestingly also resulted in a reduction of colonic nTregs, but this did however not influence the total Tregs ability to prevent colitis. *Manuscript II* investigated the role of IRF8 expressing cDC1s in AOM/DSS induced experimental colorectal cancer. Consistent with studies under homeostatic conditions, absence of IRF8 expressing cDC1s resulted in a reduced Th1 response in the colon; this however did not affect the development of colorectal tumors in AOM/DSS induced colitis.

As described in this thesis, inflammatory bowel disease is a complex and multifactorial disease. Some mutations such as IL-10R deficiency can directly lead to early onset colitis but in most patients it is a combination of genetics, dysbiosis or infection, intestinal barrier defects and sustained pro-inflammatory immunity [193, 194, 369]. The fact that I could probably have written a thesis of about 100+ pages with all the knowledge gained over the last decades about the immunology of IBD from animal models and human disease illustrates the complexity of the disease. Recent advances in the mapping of the genetic basis of disease susceptibility however offer a tool for more focused research. There is however still a gap in what role mediators such as cDCs have in the onset of IBD. To follow up on manuscript I, the next step would be to investigate the role of IRF8 expressing cDC1s in experimental T cell mediated colitis. The question however is how efficient cDC1s are at priming $CD4^+$ T cells, as they have been shown to be inferior to cDC2s at MHCII presentation [370]. cDC1s however are important producers of RA that imprints homing of T cells

to the small intestine LP and epithelium which might indicate that activated T cells in lymph nodes have reduced ability to home to the intestine [166], although this may not be applicable for homing to the colon as other chemokines have been implicated in homing of T cells to the colon. Moreover Th1 cell priming is impaired in the absence of cDC1s [166] and although Th1 cells are not the main driver of colitis, they may contribute to maintaining the pro-inflammatory environment. The significant infiltration of moDCs in the inflamed colon could indicate that once inflammation is established these moDCs have an important role in maintaining colitogenic T cells once colitis is established. CCR2 deficient mice however are still capable of inducing T cell mediated colitis despite impaired recruitment of monocytes to the intestine (unpublished results). Which may indicate that colitis establishment in T cell transfer colitis is dependent on cDC2 and possibly cDC1 priming of colitogenic T cells but that maintenance and progression of colitis occurs through the actions of moDCs or monocyte derived macrophages [142, 143].

The result that IRF8 expressing cDC1s contributed to neither anti- nor pro-tumor immunity in the AOM/DSS model of CRC was to us surprising. Our observations that the tumor environment in AOM/DSS induced CRC harbored an altered immune cell composition compared to the non-tumor tissue however indicates that the tumor environment in this model creates an immune response that favors tumor growth. Moreover the observation that cDC1s were present in decreased proportions in tumor versus non-tumor tissue could indicate that the tumor environment inhibits cDC1s' ability to prime an anti-tumor response. Future studies in this model could focus on the functionality of cDC1s in the tumor environment compared to the non-tumor environment (e.g. in *vitro* T cell stimulation assays with sorted cDC1s from tumors and non-tumor tissue). Moreover it would be of interest to investigate the roles of Th1 cells and the separate roles of nTregs and pTregs in the AOM/DSS induced CRC model.

Current treatments for IBD and CRC are not ideal as not all treatments are effective for all patient groups. Moreover, long-term effects of effective biologicals are not known and they are relatively costly. A better understanding of the immunological mechanisms and especially the mediator populations such as cDCs that potentially induce colitogenic responses is instrumental for the development of improved

treatments. The increased understanding of the underlying genetics and cross-talk of the immune system with the microbiota could assist the development of more personalized treatments. For example dendritic cell based vaccination could especially for the treatment of colorectal cancers form a more targeted treatment. DCs could be modulated in vitro with the ability to infiltrate tumors, alter the tumor induced immune-suppressive environment and induce $CD8^+$ cytotoxic T cell responses. Although we are far from realizing this concept in IBD or CRC, for some cancers such as prostate cancer or metastatic melanoma DC based vaccinations have shown some success in clinical trials [371]. In IBD there is currently a major interest in the modulation of the microbiota in order to alter the immune response in the intestine of IBD patients, clinical trials have however been implemented with mixed results [305, 316, 317]. A better understanding of a "healthy" microbiota is needed and it might be of interest to investigate interactions between cDCs and the microbiota in more detail in order to find the "bugs" that can induce the desired immune response. For example *clostridium* species are potent inducers of pTreg responses [113], but the role of cDCs in the induction of these pTregs in response to *clostridium* species has not been addressed in detail.

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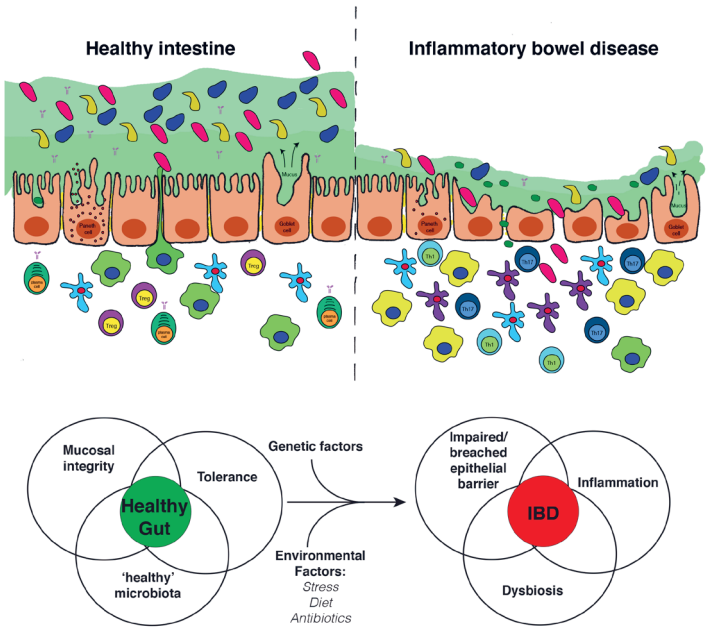

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